รายงานวิจัยฉบับสมบูรณ์

โครงการ: การศึกษาระบบไหลเวียน splanchnic เพื่อค้นหาพยาธิกำเนิดและช่วยวินิจฉัยโรคไข้เลือดออกในระยะไข้ และ การใช้อัลตราซาวด์หัวใจและ blood lactate เพื่อช่วยในการรักษาภาวะช็อกในผู้ป่วยโรคไข้เลือดออก

โดย อภิชัย คงพัฒนะโยธิน พ.บ.

พฤษภาคม 2554

สัญญาเลขที่ **RMU4980019**

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ร.ศ. น.พ. อภิชัย คงพัฒนะโยธิน (Apichai Khongphatthanayothin)

หน่วยโรคหัวใจ ภาควิชากุมารเวชศาสตร์

คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

โทรศัพท์ (02) 2564966 โทรสาร (02) 2564911

Email apichaik@yahoo.com

สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา

และสำนักงานกองทุนสนับสนุนการวิจัย

สารบัญ

Abstract 1

The Role of Vascular Endothelial Growth Factor (VEGF) Leading to Vascular Leakage in Children With Dengue Virus Infection

Patcharapa Sathupan*,**, Apichai Khongphattanayothin**, Jirabat Srisai**,***, Karakade Srikaew**,***, Yong Poovorawan**. Departments of Paediatrics, Thammasat University, Patumthani, Thailand* , Chulalongkorn University, Bangkok, Thailand** and Had Yai Hospital, Songkhla, Thailand***

Abstract

Increased vascular permeability is the main aetiology for hypovolemic shock and circulatory failure in dengue haemorrhagic fever (DHF). In this study, we investigated the role of vascular endothelial growth factor (VEGF) in the pathogenesis of DHF. Serum samples from 41 patients (15 dengue fever [DF], 26 DHF) with serologically confirmed dengue virus infection during febrile, toxic, convalescent stages and at follow-up were analyzed for VEGF. Plasma samples from additional 27 children (16 DF and 11 DHF) during febrile, toxic stages and at 4-weeks follow-up visit and 8 healthy controls were analyzed for VEGF. Serum and plasma VEGF levels were not elevated during febrile or toxic stages of dengue virus infection and were not different between patients with DF and DHF. We concluded that plasma leakage in patients with DHF cannot be explained by elevation of VEGF level during the toxic stage of the illness.

Key words: Dengue, Dengue haemorrhagic fever, Dengue shock syndrome, Vascular endothelial growth factor, Vascular leakage

บทคัดย่อที่ 1

บทบาทของ vascular endothelial growth factor ในการเกิดการรั่วของสารน้ำออกนอกเส้นเลือดในผู้ป่วยเด็กที่ติดเชื้อไวรัสเดงกี

พัชราภา สาธุพันธ์, อภิชัย คงพัฒนะโยธิน, จีราบัตร ศรีใส, การะเกด ศรีแก้ว, ยง ภู่วรวรรณ. ภาควิชากุมารเวชศาสตร์ คณะแพทยศาสตร์มหาวิทยาลัยธรรมศาสตร์, ภาควิชากุมารเวชศาสตร์ คณะแพทยศาสตร์จุฬาลงกรณ์มหาวิทยาลัย, กรุงเทพ ฯ และ กลุ่มงานกุมารเวชกรรม โรงพยาบาลหาดใหญ่, สงขลา, ประเทศไทย

บทคัดย่อ

การเพิ่มขึ้นของ vascular permeability เป็นสาเหตุหลักของการเกิดช็อกจากการขาดสารน้ำในเส้นเลือดในผู้ป่วยโรคไข้เลือดออก

วัตถุประสงค์: เพื่อหาว่า vascular endothelial growth factor (VEGF) มีความเกี่ยวข้องกับพยาธิกำเนิดของโรคไข้เลือดออกหรือไม่

ระเบียบการวิจัย: หาระดับ VEGF ในซีรั่มของผู้ป่วยจำนวน 41 คน (ไข้เดงกี 15 คน, ไข้เลือดออก 26 คน) ซึ่งได้รับการยืนยันการติดเชื้อเดงกีโดยวิธีทางน้ำเหลืองวิทยา ในระยะไข้, ระยะวิกฤต, ระยะฟื้นตัว และหลังจากหายจากโรค หาระดับ VEGF ในพลาสมาในผู้ป่วยอีก 27 ราย (ไข้เดงกี 16 คน, ไข้เลือดออก 11 คน) ในระยะไข้, ระยะพักฟื้น, และหลังจากหายจากโรคเป็นเวลา 4 สัปดาห์ และในเด็กปกติ 8 ราย

ผลการศึกษา: ระดับ VEGF ในซีรั่มและพลาสมาในผู้ป่วยติดเชื้อเดงกีไม่ได้สูงกว่าระดับเมื่อผู้ป่วยหายจากโรค และ/หรือ ระดับ VEGF ในเด็กปกติอย่างมีนัยสำคัญ และไม่มีความแตกต่างของระดับ VEGF ในผู้ป่วยไข้เลือดออกเมื่อเทียบกับผู้ป่วยไข้เดงกี

สรุปผลการศึกษา: การรั่วของสารน้ำออกนอกเส้นเลือดในผู้ป่วยไข้เลือดออกไม่สามารถอธิบายได้โดยการเพิ่มขึ้นของ VEGF

คำสำคัญ ไข้เลือดออก, ไข้เดงกี, ช็อก, ระบบไหลเวียน, vascular endothelial growth factor, VEGF

Abstract 2

Matrix Metalloproteinase-9 (MMP-9) in Children with Dengue Virus Infection

Nipasiri Voraphani[1], Apichai Khongphattanayothin[1], Karakade Srikaew[1,2], Praewpilai Tontulawat[1], Yong Poovorawan[1]*

Departments of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand[1], and Hadyai Hospital, Songkhla, Thailand[2]

Abstract

The purpose of this study was to investigate the role of MMP-9 in the pathogenesis of vascular leakage in patients with dengue virus infection. Serum samples from 24 children with serologically confirmed dengue virus infection [dengue fever (DF) = 16, dengue hemorrhagic fever (DHF) = 8, age 9.5± 2.4 years, 67% male] during the febrile, toxic stages, and at follow-up were analyzed for MMP-9. Serum samples obtained from 7 healthy children served as the control group. In patients with dengue virus infection, serum MMP-9 was lower at the febrile (227.0 ± 186.9 ng/ml) and toxic stages (150.9 ± 151.7) compared to at follow-up (424.5 ± 227.8 ng/ml) and the control group (393.3 ± 125.9 ng/ml, $p < 0.001$ by one-way ANOVA). There was no significant difference between MMP-9 levels in patients with DHF and those with DF at all stages of the disease. In conclusion, serum MMP-9 level decreased during the febrile and toxic stages of dengue virus infection.

Key Words: Dengue, Dengue hemorrhagic fever, MMP-9

บทคัดย่อที่ 2

การศึกษาระดับ Matrix Metalloproteinase-9 ในผู้ป่วยเด็กที่ติดเชื้อไวรัสเดงกี

นิภาศิริ วรปาณิ[1], อภิชัย คงพัฒนะโยธิน[1], การะเกด ศรีแก้ว[1, 2], แพรวพิไล ตัณฑุลาวัฒน์[1], ยง ภู่วรวรรณ[1]
[1]ภาควิชากุมารเวชศาสตร์ คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย, กรุงเทพมหานคร
[2]โรงพยาบาลหาดใหญ่, สงขลา

บทคัดย่อ

ความสำคัญและที่มาของปัญหาการวิจัย:
ในผู้ป่วยที่ติดเชื้อไวรัสเดงกี vascular leakage เป็นสิ่งสำคัญที่ใช้ในการแยก dengue hemorrhagic fever (DHF) ออกจาก dengue fever (DF) Matrix metalloproteinase-9 (MMP-9) เป็น enzyme ที่มีผลต่อการย่อยสลายของ extracellular matrix และการเกิด endothelial injury โดยพบว่ามีส่วนร่วมในการเกิด vascular diseases
วัตถุประสงค์ของการวิจัย:
เพื่อศึกษาความสัมพันธ์ระหว่างระดับ MMP-9 กับความรุนแรงของโรค ในผู้ป่วยที่ติดเชื้อไวรัสเดงกี
วิธีการวิจัย:
ตัวอย่าง serum ของผู้ป่วยเด็กที่ได้รับการยืนยันว่าติดเชื้อไวรัสเดงกี 24 คน (16 DF, 8 DHF) ในระยะไข้, ระยะวิกฤติ, และเมื่อมาติดตามการรักษา ได้ถูกนำมาวิเคราะห์หาระดับ MMP-9 โดยมี serum ของเด็กปกติ 7 คน เป็นกลุ่มควบคุม
ผลการวิจัย:
ในผู้ป่วยที่ติดเชื้อไวรัสเดงกี ระดับ MMP-9 ในระยะในระยะไข้และระยะวิกฤติ ต่ำกว่าในช่วงที่มาติดตามการรักษา และต่ำกว่ากลุ่มควบคุม ($p<0.001$) ไม่มีความแตกต่างกันของระดับ MMP-9 ระหว่างผู้ป่วย DF กับ DHF ในระยะในระยะ ไข้, ระยะวิกฤติ, และเมื่อมาติดตามการรักษา
สรุปการวิจัย:
ระดับ serum MMP-9 ลดลงในระยะ febrile stage และ toxic stage ของการติดเชื้อไวรัสเดงกี
คำสำคัญ: ไข้เดงกี, ไข้เลือดออก, MMP-9

Abstract 3

Increased Level of Hepatocyte Growth Factor in Children with Dengue Virus Infection

NIPASIRI VORAPHANI[1], APIRADEE THEAMBOONLERS[1], APICHAI KHONGPHATTHANAYOTHIN[1], CHIRABAT SRISAI[1,2], YONG POOVORAWAN[1*]

Department of Paediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand[1], and Hadyai Hospital, Songkhla, Thailand[2]

Abstract

Background: Evidence of hepatocellular damage is common in dengue-infected individuals. Hepatocyte growth factor (HGF), a key cytokine responsible for liver regeneration, may play a prognostic role in dengue virus infection.

Aim: To determine the relationship between serum HGF level and disease severity in patients with dengue virus infection.

Methods: Serum samples from 27 children [17 dengue fever (DF), 10 dengue haemorrhagic fever (DHF)] with serologically confirmed dengue virus infection during the febrile, toxic stages, and at follow-up were analysed for HGF. Serum samples obtained from 9 healthy children served as the control group.

Results: In dengue-infected patients, serum HGF was significantly higher at the febrile and toxic stages than at follow-up ($p<0.05$). In comparison with DF, patients with DHF had a greater level of HGF at the febrile stage ($p<0.05$). A cut-off HGF level of 1,220 pg/mL obtained during the febrile stage showed a sensitivity of 90%, and a specificity of 53%, for predicting the clinical progression to DHF (area under the ROC curve = 0.75).

Conclusion: Serum HGF level at the early stage of dengue virus infection is elevated and may be a useful predictor for clinical progression to DHF.

Keyword: Hepatocyte growth factor, Children, Dengue, Dengue haemorrhagic fever

บทคัดย่อที่ 3

การศึกษาระดับ hepatocyte growth factor ในผู้ป่วยเด็กที่ติดเชื้อไวรัสเดงกี

นิภาศิริ วรปาณิ[1], อภิรดี เทียมบุญเลิศ[1], อภิชัย คงพัฒนะโยธิน[1], จีราบัตร ศรีใส[1, 2], ยง ภู่วรวรรณ[1]
[1]ภาควิชากุมารเวชศาสตร์ คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย, กรุงเทพมหานคร
[2]โรงพยาบาลหาดใหญ่, สงขลา

บทคัดย่อ

ความสำคัญและที่มาของปัญหาการวิจัย:
ในผู้ป่วยที่ติดเชื้อไวรัสเดงกีพบว่ามีความผิดปกติของตับได้บ่อย โดยใน dengue hemorrhagic fever (DHF) จะมีระดับความรุนแรงมากกว่า dengue fever (DF) Hepatocyte growth factor (HGF) เป็น cytokine หลักที่สำคัญต่อ liver regeneration หลังจากที่มีการทำลายของเนื้อตับ และอาจมีบทบาทในการช่วยพยากรณ์โรคในผู้ป่วยที่ติดเชื้อไวรัสเดงกี
วัตถุประสงค์ของการวิจัย:
เพื่อศึกษาความสัมพันธ์ระหว่างระดับ serum HGF กับความรุนแรงของโรค ในผู้ป่วยที่ติดเชื้อไวรัสเดงกี
วิธีการวิจัย:
ตัวอย่าง serum ของผู้ป่วยเด็กที่ได้รับการยืนยันว่าติดเชื้อไวรัสเดงกี 27 คน (17 DF, 10 DHF) ในระยะไข้, ระยะวิกฤติ, และเมื่อมาติดตามการรักษา ได้ถูกนำมาวิเคราะห์หาระดับ HGF โดยมี serum ของเด็กปกติ 9 คน เป็นกลุ่มควบคุม
ผลการวิจัย:
ในผู้ป่วยที่ติดเชื้อไวรัสเดงกี ระดับ serum HGF ในระยะไข้และระยะวิกฤติสูงกว่าเมื่อมาติดตามการรักษา ($p<0.05$) ผู้ป่วย DHF มีระดับ serum HGF ในระยะไข้สูงกว่าในผู้ป่วย DF ($p<0.05$) ระดับ HGF ที่สูงกว่า 1,220 pg/mL ในระยะไข้ช่วยพยากรณ์การดำเนินโรคไปเป็น DHF โดยมีความไว 90% และความจำเพาะ 53% (area under the ROC curve 0.75)
สรุปการวิจัย:
ในผู้ป่วยที่ติดเชื้อไวรัสเดงกี ระดับ serum HGF ที่สูงขึ้นในระยะ febrile stage อาจเป็นตัวบ่งชี้ที่ช่วยพยากรณ์การดำเนินโรคไปเป็น DHF

คำสำคัญ: Hepatocyte growth factor, เด็ก, ไข้เดงกี, ไข้เลือดออก

Abstract 4

Capillary Lactate as a Potential Hemodynamic Monitoring Tool in Patients with Dengue Virus Infection

Nawarat Rungteeranon*, Pentip Supachokechaiwattana**, Kanyalak Vithessonthi*, Vidhavas La-orkhun*, Pornthep Lertsapcharoen*, Apichai Khongphatthanayothin*.
Departments of Paediatrics, Faculty of Medicine, Chulalongkorn University* and Sawanpracharak Hospital**, Bangkok and Nakorn Sawan, Thailand.

Abstract

To study capillary lactate as hemodynamic monitoring tool in patients with dengue virus infection, lactate was determined within 6 hours of defervescence and 12 hours thereafter in 27 patients (age 10.4±3.0 years). All patients with normal initial lactate (1.8±0.3 mmol/L, n=11) received intravenous fluid of less than 650 mL/m^2 in the next 12 hours and none had pleural effusion >5% at convalescence. Among patients with high initial lactate (3.0±0.5 mmol/L, n=16), 5 (31%) had pleural effusion >5%, all of whom appeared to have relatively rapid decline of lactate. The amount of fluid administration correlated to the rate of lactate decline ($r=-0.42$, $p<0.05$). We conclude that patients with dengue virus infection with normal lactate at the initial hours after defervescence generally have mild clinical presentation. In patients with high initial lactate, subsequent follow-up level has the potential to identify the patients who received under- or over-treatment and should be further studied.

Key words**:** Dengue, Dengue hemorrhagic fever, Lactate, Hemodynamic monitoring, Children

บทคัดย่อที่ 4

การใช้ capillary lactate เพื่อตรวจติดตามสภาวะไหลเวียนในผู้ป่วยไข้เลือดออก

นวรัตน์ รุ่งธีรานนท์*, เพ็ญทิพย์ ศุภโชคชัยวัฒนา**, กัญญลักษณ์ เศรษฐไกรกุล*, วิทวัส ลออคุณ*, พรเทพ เลิศทรัพย์เจริญ* ภาควิชากุมารเวชศาสตร์ คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย* และกลุ่มงานกุมารเวชกรรม โรงพยาบาลสวรรค์ประชารักษ์

บทคัดย่อ

ผู้วิจัยได้ทำการตรวจ capillary lactate ในผู้ป่วยติดเชื้อเดงกี 27 ราย (อายุ 10.4 ±3.0 ปี) ภายใน 6 ชั่วโมงหลังจากไข้ลดและ 12 ชั่วโมงถัดไป ผู้ป่วยที่มีค่า lactate ที่ตรวจครั้งแรกอยู่ในเกณฑ์ปกติ (1.8±0.3 mmol/L, n=11) ได้รับสารน้ำทางหลอดเลือดดำน้อยกว่า 650 ml/m^2 ใน 12 ชั่วโมงถัดมา และไม่มีผู้ป่วยคนใดมีน้ำในโพรงเยื่อหุ้มปอดด้านขวามากกว่า 5% ในระยะพักฟื้น ส่วนผู้ป่วยที่มีค่า lactate ครั้งแรกผิดปกติ (3.0 ± 0.5 mmol/L, n=16) มีผู้ป่วย 5 คน (31%) ที่มีน้ำในโพรงเยื่อหุ้มปอดมากกว่า 5% โดยทั้งหมดดูเหมือนว่าจะเป็นผู้ป่วยที่มีการลดลงของ lactate ค่อนข้างเร็วกว่าผู้ป่วยที่เหลือ ปริมาณสารน้ำที่ได้รับมีความสัมพันธ์โดยตรงกับการลดลงของ lactate ($r=0.42$, $p < 0.05$) โดยสรุป ผู้ป่วยติดเชื้อไข้เลือดออกที่มีค่า lactate ปกติเมื่อเข้าสู่ระยะวิกฤติ ส่วนใหญ่จะมีอาการไม่รุนแรง ส่วนในผู้ป่วยที่ค่า lactate สูงผิดปกติ การตรวจติดตามค่า lactate อาจมีประโยชน์ในการติดตามผู้ป่วยว่าได้รับสารน้ำมากหรือน้อยไปหรือไม่ และควรได้รับการศึกษาต่อไป

คำสำคัญ: เดงกี, ไข้เลือดออก, lactate, การตรวจติดตามระบบไหลเวียน, เด็ก

Abstract 5

Relationship between portal blood flow and liver enzyme elevation in patients with dengue virus infection: Is liver injury a result of hepatic hypoperfusion?

Apichai Khongphatthanayothin, Pasjutha Ratanasukol, Pentip Supachokechaiwattana, Kanyalak Sethkrikul, Pornthep Lertsapcharoen.
Department of Pediatrics, Faculty of Medicine, Chulalongkorn University and King Chulalongkorn Memorial Hospital, Bangkok, Thailand

Abstract

To find out if liver injury in patients with dengue virus infection is caused by ischemia (ischemic hepatitis), we studied portal blood flow in 36 serologically-confirmed dengue virus infection during the toxic stage. The patients' age was 10.9±2.5 years and 13 had dengue fever, 10 dengue hemorrhagic fever and 13 dengue shock syndrome. Plotting of ultrasound-derived portal blood flow against the level of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) demonstrate a "threshold effect" of portal venous blood flow to the elevation of liver enzyme. All patients with significantly elevated AST and ALT (> 200 IU/mL) had portal venous flow of less than 300 mL/min/m^2. Some patients, however, was protected from liver injury despite low portal venous blood flow. We concluded that liver injury in patients with dengue virus infection could be partly explained by ischemia, with the possibility of other factors contributing to the different degree of liver injury. Alteration of the splanchnic circulation may be an important pathophysiologic process in patients with dengue virus infection.

Key Words**:** Dengue, Dengue hemorrhagic fever, Liver, Splanchnic circulation, Children

บทคัดย่อที่ 5

ความสัมพันธ์ระหว่างอัตราการไหลของเลือดใน portal vein กับระดับของ liver enzyme ในผู้ป่วยติดเชื้อเดงกี

อภิชัย คงพัฒนะโยธิน, ภาสจุฑา รัตนสุคลธ์, เพ็ญทิพย์ ศุภโชคชัยวัฒนา, กัญญลักษณ์ เศรษฐไกรกุล, พรเทพ เลิศทรัพย์เจริญ

บทคัดย่อ

ผู้วิจัยได้ทำการวัดอัตราการไหลของเลือดใน portal vein ในผู้ป่วย 36 คน ที่ได้รับการยืนยันทางน้ำเหลืองวิทยาว่าติดเชื้อไวรัสเดงกีในระยะวิกฤติ ผู้ป่วยมีอายุเฉลี่ย 10.9 $\pm$ 2.5 ปี โดย 13 คนเป็นไข้เดงกี, 10 คนเป็นไข้เลือดออกที่ไม่ช็อก และ 13 คนมีภาวะช็อก การพลอตอัตราการไหลของเลือด กับ aspartate aminotransferase (AST) และ alanine aminotransferase (ALT) พบว่ามี “threshold effect” ของอัตราการไหลของเลือดใน portal vein กับการเพิ่มขึ้นของ liver enzyme โดยผู้ป่วยทุกรายที่มี AST และ/หรือ ALT เพิ่มขึ้นมากกว่า 200 IU/ml มีอัตราการไหลของเลือดใน portal vein ที่ต่ำกว่า 300 ml/min/m^2 แต่มีผู้ป่วยบางรายที่ไม่มีการเพิ่มขึ้นของ liver enzyme แม้จะมีอัตราการไหลของเลือดที่ต่ำกว่าค่านี้ สรุปว่าการเพิ่มขึ้นของ liver enzymeในผู้ป่วยที่ติดเชื้อเดงกีที่สามารถอธิบายได้บางส่วนจากการลดลงของอัตราการไหลของเลือดใน portal vein ทั้งนี้ยังอาจมีปัจจัยอื่นที่มีผลต่อการเกิดอันตรายต่อตับ การเปลี่ยนแปลงของ splanchnic circulation อาจจะเป็นพยาธิกำเนิดที่สำคัญของโรคที่เกิดจากการติดเชื้อไวรัสเดงกี

คำสำคัญ: เดงกี, ไข้เลือดออก, ตับ, ระบบไหลเวียน splanchnic, เด็ก

Abstract 6

Correlation between echocardiographic derived hemodynamic variables and capillary lactate during toxic stage of dengue hemorrhagic fever

Sarawut Pinta, Soravit Pongpittayut, Kanyalak Sethkrikul, Vithawas La-ookhun, Pornthep Lertsapcharoen, Apichai Khongphatthanayothin.

Department of Pediatrics, Faculty of Medicine, Chulalongkorn University and King Chulalongkorn Memorial Hospital, Bangkok, Thailand

Abstract

To find the usefulness of a single echocardiographic determination of the hemodynamic status at the toxic stage for predicting the adequacy of the circulation in patients with dengue virus infection, 20 patients (12 boys and 8 girls, mean age 11.0 ± 2.9 years) were enrolled in this study. Echocardiographic determinations of cardiac index and end-diastolic volume index were performed within 6 hours of defervescence and data were correlated with 1) capillary lactate at the time of echocardiogram, 2) capillary lactate 12 hours later, and 3) the change of capillary lactate within the 12 hours. Twelve patients had dengue fever, 5 had dengue hemorrhagic fever without shock and 3 had dengue shock syndrome. No correlation was found between the cardiac index or end-diastolic volume index and lactate/change of lactate in this group of patient. It was concluded that single determinations of cardiac index or end-diastolic volume index in patients with dengue virus infection at toxic stage were not predictive of the lactate at that time nor the change thereafter.

บทคัดย่อที่ 6

ความสัมพันธ์ระหว่างค่าตัวแปรทางระบบไหลเวียนซึ่งวัดโดยคลื่นเสียงสะท้อนหัวใจกับระดับของ capillary lactate ในผู้ป่วยติดเชื้อไวรัสเดงกีในระยะช็อก

ศราวุฒิ ปินตา, สรวิทย์ พงษ์พิทยายุทธ, กัญญลักษณ์ เศรษฐไกรกุล, วิทวัส ลออคุณ, พรเทพ เลิศทรัพย์เจริญ, อภิชัย คงพัฒนะโยธิน
ภาควิชากุมารเวชศาสตร์ คณะแพทย์ศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

บทคัดย่อ

ผู้วิจัยได้ทำการวัดค่าตัวแปรทางระบบไหลเวียนในผู้ป่วยเด็กจำนวน 20 ราย (ชาย 12 คน หญิง 8 คน, อายุเฉลี่ย 11.0 ± 2.9 ปี) เพื่อหาว่าตัวแปรเหล่านี้มีความสัมพันธ์กับความเพียงพอของระบบไหลเวียนซึ่งวัดโดย capillary lactate ในผู้ป่วยที่ติดเชื้อ dengue virus ในระยะวิกฤติหรือไม่ การวัดค่าตัวแปรทางระบบไหลเวียนทำโดยคลื่นเสียงสะท้อนหัวใจภายใน 6 ชั่วโมงหลังไข้ลง ส่วน capillary lactate ทำภายใน 6 ชั่วโมงหลังไข้ลง และ หลังจากนั้นอีก 12 ชั่วโมง ผลการวิจัยไม่พบว่ามีความสัมพันธ์ระหว่าง cardiac index หรือ end-diastolic volume index กับ capillary lactate ในขณะนั้น หรือ 12 ชั่วโมงถัดมา และไม่พบความสัมพันธ์ระหว่าง cardiac index หรือ end-diastolic volume index กับการเปลี่ยนแปลงของ capillary lactate ใน 12 ชั่วโมงหลังจาก echocardiogram ผู้วิจัยสรุปว่าการวัดค่าตัวแปรทางระบบไหลเวียนโดย ultrasonography หนึ่งครั้งในผู้ป่วยที่ติดเชื้อไข้เลือดออกในภาวะวิกฤติไม่มีความสัมพันธ์กับ capillary lactate ในขณะนั้นและไม่สามารถทำนายการเปลี่ยนแปลงของ capillary lactate ใน 12 ชั่วโมงถัดไปได้

Abstract 7

Reversed Direction of Portal Venous Blood Flow in a Patient with Dengue Virus Infection and Fatal Liver Failure

Apichai Khongphatthanayothin, M.D., Atchara Mahayosnond, M.D., Yong Poovorawan, M.D.

Abstract

We reported a 10-year old boy with dengue shock syndrome and fatal liver failure. Ultrasonogram of the live and portal veins during toxic stage showed reversed direction of portal blood flow. We postulated that hepatic sinusoidal obstruction of portal blood flow may be the cause of liver failure in children with dengue hemorrhagic fever.

บทคัดย่อที่ 7

การไหลย้อนทางของ portal blood flow ในผู้ป่วยไข้เลือดออกที่มีภาวะตับวาย

อภิชัย คงพัฒนะโยธิน, อัจฉรา มหายศนันท์, ยง ภู่วรวรรณ

บทคัดย่อ

ผู้วิจัยได้รายงานผู้ป่วยอายุ 10 ปีที่มีภาวะช็อกและตับวายจากไข้เลือดออก การตรวจตับและ portal vein ด้วยคลื่นเสียงสะท้อนพบว่าเลือดใน portal vein มีการไหลย้อนทางในขณะที่ผู้ป่วยอยู่ในภาวะวิกฤติของไข้เลือดออก ผู้วิจัยสันนิษฐานว่า hepatic sinusoidal obstruction อาจเป็นพยาธิสภาพของการเกิดภาวะตับวายในผู้ป่วยไข้เลือดออก

Executive Summary

Dengue virus infection is one of the most important emerging infectious diseases in Thailand and the world[1, 2]. Vascular leakage, low cardiac output and shock develop in some patients infected with dengue virus (dengue hemorrhagic fever, DHF) which may lead to morbidity and mortality. The pathogenesis of shock in DHF is not entirely understood. Although increased capillary permeability is believed to be the main etiology for shock, a recent study failed to demonstrate difference of capillary permeability between patients with and without shock[3]. Apart from increased capillary permeability, we recently demonstrated that splanchnic venous pooling (measured by portal vein congestion index or PVCGI) during the toxic stage was an important mechanism for low cardiac output and shock in DHF [4]. In this patient cohort, PVCGI at toxic stage of > 0.065 had 86% sensitivity and 81% specificity in predicting pleural effusion (leakage) at convalescent stage. Whether this splanchnic venous pooling occurs earlier (in febrile stage) and if so, could it be used to predict which patient would develop DHF (or vascular leakage) were not known.

Patients with dengue virus infection generally present with febrile illness which typically lasts for 3-9 days. At the time of defervescence, some patients infected with dengue virus develop vascular leakage (DHF) which is a more severe form of the disease compared to dengue fever (DF). Up to now, vascular leakage in patients with dengue virus infection cannot be predicted with certainty during the febrile period and its mechanism is still largely unknown. Rising of hematocrit and decreasing platelet number are often used clinically to determine which patient has DHF, but these findings are generally not seen until defervescence. The first part of this study was done to determine if the circulatory indices of splanchnic vascular system and/or changes of cytokines-vascular endothelial growth factor (VEGF) and hepatic growth factor (HGF)-during the febrile stage could predict which patient would develop DHF as opposed to DF. Studies of the systemic circulatory indices, various selected laboratory indices were also undertaken either in the same or different patient cohort to evaluate the predictive value of these variables for the diagnosis of DHF.

The cause(s) of this splanchnic venous pooling is(are) not clear but one potential explanation was that dengue virus infection of hepatic endothelial and Kupffer cells[5] caused narrowing of hepatic sinusoid and back-pressure into the splanchnic circulation, similar to what had been described as “sinusoidal obstruction syndrome or SOS” of the liver induced by certain toxins or chemotherapeutic agents[6]. We postulate that, in DHF, sinusoidal obstruction occured and shared the same mechanism as the early phase of SOS. Vasoconstrictors (such as endothelin-1[6-8]) and protease (such as matrix metalloproteinase or MMP[9]) have been implicated in the mechanism of SOS by causing sinusoidal constriction and injury to extracellular matrix causing endothelial cell swelling and dislodgement from the basement membrane[9, 10], respectively. The role of these agents in the pathogenesis of DHF has never been previously studied. Because of technical difficulties in the study of endothelin-1, we elected to study MMP-2 and MMP-9 during the febrile and toxic stage of patients with dengue infection. Patients at King Chulalongkorn Memorial Hospital and Had-Yai Hospital were enrolled for these studies.

Liver involvement in patients with dengue virus infection is well known. Liver enzyme elevation was noted in 45-98% of patients with dengue virus infection[11, 12]. Liver failure due to dengue virus infection is well documented in the literature[13] and dengue virus is the most common cause of liver failure in Thai children in the current era[14].

The pathogenesis of liver involvement in DHF and DF is not well understood. Potential explanation includes direct infection of hepatocyte[15], hypoperfusion of liver from shock, and sinusoidal endothelial damage[16]. Because liver enzyme elevation tended to be more severe in patients with higher severity of the illness (i.e. in shock cases more than non-shock cases), we hypothesized that hepatic hypoperfusion may be the etiology for liver injury in this disease. The objective of this study was to correlate the degree of hepatic hypoperfusion with that of liver injury. Because portal vein supplies the majority of hepatic blood flow and its ease of measurement compared to hepatic arterial flow, we use portal blood flow as the surrogate for hepatic perfusion. Liver enzymes (alanine aminotransferase, ALT and aspartate aminotransferase, AST) were used to quantify the degree of livery injury.

The most important treatment of DHF is intravenous fluid. Because of increased vascular permeability, overly treated patients may develop fluid overload (such as pleural effusion, pulmonary edema and ascites) as a result. Because clinical examination may be unreliable to guide fluid management in some patients, such as in obese patients, other means of monitoring fluid management in these patients with dengue shock syndrome is desirable. Goal-directed therapy using invasive and non-invasive hemodynamic monitorings in conjunction with clinical data has been shown to improve the outcome of treatment in other etiologies of shock[17-20]. Although invasive monitoring such as the use of central venous catheter has been recommended in the treatment of certain patients with dengue shock syndrome[21], its used is not without complication due to low platelet count and coagulopathy in this disease. The original idea for the part 2 of this study was to evaluate the value of cardiac ultrasound for hemodynamic monitoring of children with DHF. But as the results of ultrasonographic monitoring of hemodynamic data were not encouraging (see abstract 6, page 12,13 and text page 87-96), we investigated the role of using blood lactate measurement by a hand-held device for hemodynamic monitoring in patients with dengue virus infection. Lactate has been evaluated and used to monitor the hemodynamic status in patients with bacterial sepsis and/or septic shock[22, 23], but no such study was available for children with dengue virus infection or DHF/DSS. Because of it ease of use and low cost, lactate measurement is more likely to be used in the real clinical setting than echocardiography. The study of lactate for hemodynamic monitoring was done from patient cohorts at King Chulalongkorn Memorial Hospital and Sawanpracharak Hospital, Nakorn Sawan.

This project comprised 3 patients cohorts in which several observational and case-control studies were performed to further understand the pathogenesis and treatment of dengue virus infection and dengue hemorrhagic fever (DHF). Focus was especially made for the role of hepatosplanchnic circulation and the liver in the pathogenesis of DHF. Non-invasive hemodynamic monitoring using echocardiogram and blood lactate in patients with dengue virus infection was also evaluated.

Objectives

1. To study the utility of using splanchnic circulatory indices, systemic circulatory indices, cytokines (as described in objective 2), and selected laboratory indices as a predictor for DHF/DSS (during febrile and toxic stage).
2. To study the association between the severity of dengue virus infection and selected cytokines (vascular endothelial growth factor, VEGF and hepatocyte growth factor, HGF) and pretease (MMP-2 and MMP-9).
3. To study the utility of using blood lactate as hemodynamic monitoring for low cardiac output syndrome and shock in patients with dengue virus infection.
4. To study the relationship between ultrasonographic-derived portal vein blood flow and liver enzyme elevation in patients with dengue virus infection

Methods

1. Subjects

Different cohorts of patients were enrolled as follow.

Cohort 1 (for objectives 1 and 4): 120 patients with serological or PCR-confirmed dengue virus infection at King Chulalongkorn Memorial Hospital.

Cohort 2 (for objectives 2): 27 patients with serological or PCR-confirmed dengue virus infection at Had-Yai Hospital.

Cohort 3 (for objectives 3): 27 patients with serological or PCR-confirmed dengue virus infection at King Chulalongkorn Memorial Hospital and Sawanpracharak Hospital, Nakorn Sawan.

2. Procedures

Cohort 1: Ultrasonography of the portal vein size, blood flow velocity and portal vein congestion index (PVCGI) at febrile stage and toxic stage. Measurement of systemic circulatory indices by echocardiogram (cohort 1). A convenient sample of blood determination of ALT and AST were done in 36 patients in this group.

Cohort 2: Blood collections for determination of cytokines, MMP, other selected laboratory data and serologic tests for dengue virus infection during febrile, toxic stages and at follow-up. Determinations of VEGF, HGF, MMP-2 and MMP-9 were be done by ELISA method at Hepatitis Virus Research Laboratory, Faculty of Medicine, Chulalongkorn University.

Cohort 3: Determination of capillary lactate by a hand-held device (Acctrend lactate, Roache Diagnostic, Germany) at febrile stage, toxic stage (within 6 hours) and 12 hours thereafter.

3. Analysis:

Cohort 1 and 2 (objectives 1 and 2): Comparing splanchnic circulatory indices, systemic circulatory indices, VEGF, MMP-2, MMP-9, HGF and other selected laboratory data at febrile stage and toxic stages between patients with DF and DHF. If significant difference was found in any variable between patients with DF and DHF, a receiver operative characteristic (ROC) curve for that particular variable as a predictor for DHF was then constructed and its cut-off value, sensitivity and specificity were then determined.

Cohort 3 (for objective 3): Analysis of the patient outcome based on the level of lactate determined just after defervescence (early into the toxic stage). Analyze the relationships between the change of lactate level and the amount of intravenous fluid resuscitation and pleural effusion index after the treatment (at convalescent stage)

Cohort 1 (for objective 4): In 36 patients in whom ALT and AST data were available, untrasonographic-derived portal vein blood flow was plotted against the ALT and AST.

4. Clinical management of the patients

Diagnosis and grading of DF/DHF were done according to the criteria published by the World Health Organization (WHO)[21]. ELISA test for dengue virus infection was done at the Arm Force Research Institute of Medical Sciences (AFRIMS). Diagnosis of DHF require at least 1 evidence of capillary leakage (rising of hematocrit > 20% or presence of pleural effusion and/or ascites). DSS was diagnosed when the patient with DHF had clinical findings

of shock as: 1) hypotension for age and cold clammy skin or restlessness or 2) narrow pulse pressure (<= 20 mmHg.) and rapid and weak pulse[21]. Ultrasonographic study of the right pleural cavity was used to detect the presence of pleural effusion during the convalescent stage in all cases. The classification of the patient's clinical severity was done by the agreement of two physicians who were blinded to the ultrasonographic/echocardiographic and/or lactate data. Treatments including fluid administration of all patients were given by house staff and attending staff of our department according WHO guideline[21].

5. Ethic committee approval

The study was approved by the Ethic Committee of the Faculty of Medicine, Chulalongkorn University and other participating centers. Written informed consent was obtained from each subject and/or appropriate guardian prior to enrollment.

Results

1. Patients demographic data

Cohort 1 consisted of 120 patients with serologically or PCR-proven dengue virus infection from King Chulalongkorn Memorial Hospital. Ultrasonographic assessment of splanchnic circulatory system could be done in 57 patients at toxic stage and 22 patients during the febrile stage. Various variables of the systemic circulatory system and selected laboratory data were obtained in some patients (Table 2). The demographic and clinical data of these patients are summarized in Table 1-1 as follow:

Table 1-1: Demographic and clinical data of 120 patients enrolled.

	DF (n = 59)	DHF (n = 44)	DSS (n = 27)	*p*-value
Age (years)	9.9 ± 2.7	11.4 ± 2.8	10.4±3.0	< 0.05
Sex (M/F)	25/24	27/17	15/12	NS
Weight (Kg)	31.7 ± 14.0	39.9 ± 16.6	37.1 ± 13.9	< 0.05
Maximal Hematocrit (%)	40.3 ± 3.6	45.1 ± 3.9	47.5 ± 5.0	< 0.001
Lowest Platelet ($x10^9$/L)	89.8 ± 40.6	43.3 ± 27.7	31.6 ± 21.4	< 0.001

NS = not significant

Cohort 2 consisted of 27 patients with with serologically or PCR-proven dengue virus infection from Had-Yai Hospital. The demographic and clinical data are summarized in Table 1-2 as follow:

Table 1-2: Demographic and clinical data of 27 patients enrolled for cytokines/MMP study.

	DF (n = 17)	DHF+DSS (n = 10)	*p*-value
Age (years)	9.7 ± 2.4	9.9 ± 2.8	NS
Sex (M/F)	9/8	9/1	NS
Body temperature max (C)	39.6 ± 1.0	39.7 ± 0.5	NS
Maximal Hematocrit (%)	41.0 ± 3.9	45.5 ± 4.0	< 0.01
Lowest Platelet ($x10^9$/L)	86 ± 40	74 ± 70	NS

NS = not significant

Cohort 3 consisted of 27 patients with with serologically or PCR-proven dengue virus infection from Chulalongkorn and Sawanpracharak Hospitals. The demographic and clinical data are as summarized in Table 1-3 as follow:

Table1-3: The demographic and clinical data of 27 patients enrolled for lactate study.

DF ;dengue fever, DHF ;dengue hemorrhagic fever, Hct ;hematocrit, Plt; platelet number, PEI; pleural effusion index

	DF (n = 12)	DHF (n = 11)	DSS (n = 4)	p-value
Sex (M:F)	9:3	4:7	4:0	0.04
Age (yr)	11.4±3.1	9.6±2.8	9.7±3.1	0.38
Body weight (Kg)	46.8±19.0	33.1±15.2	39.3±9.0	0.16
Duration of fever (days)	5.0±0.6	4.7±0.9	4.8±1.0	0.69
Maximum Hct (%)	41.2±2.7	45.4±3.1	45.0± 4.7	0.01
Lowest Plt ($x10^3/mm^3$)	61.8±22.9	49.3±26.7	61.3±33.4	0.49
PEI (mm)	0	7.1±9.8	7.5±10.7	0.06
Lactate level during toxic stage	2.2±0.6	2.6±0.6	3.2±0.6	0.03

2. The differences of each variables between patients with dengue fever (DF) and dengue hemorrhagic fever (DHF) are shown in Table 2 (page 28)

For those variables that demonstrated significant differences, an ROC curve was constructed to determine the area under the ROC curve and cutoff with sensitivity and specificity to differentiate patients with DF from DHF (Table 2, page 28). The variables that showed significant difference during the toxic stage are printed in blue and those variables that showed difference during the febrile stage are printed in red. Particular attention was made to those variables that showed difference during the febrile stage because these variables had the potential to be an early marker for DHF and may shed light into the early pathophysiologic process/mechanism that make certain patients infected with dengue virus progressed to DHF rather than DF. The variables that showed early changes during the febrile stage in patient with DHF compared to DF were hepatocyte growth factor (HGF) and prothrombin time (PT), with ALT showed a trend toward significance ($p = 0.07$).

3. The value of lactate in the monitoring of patients with dengue virus infection:

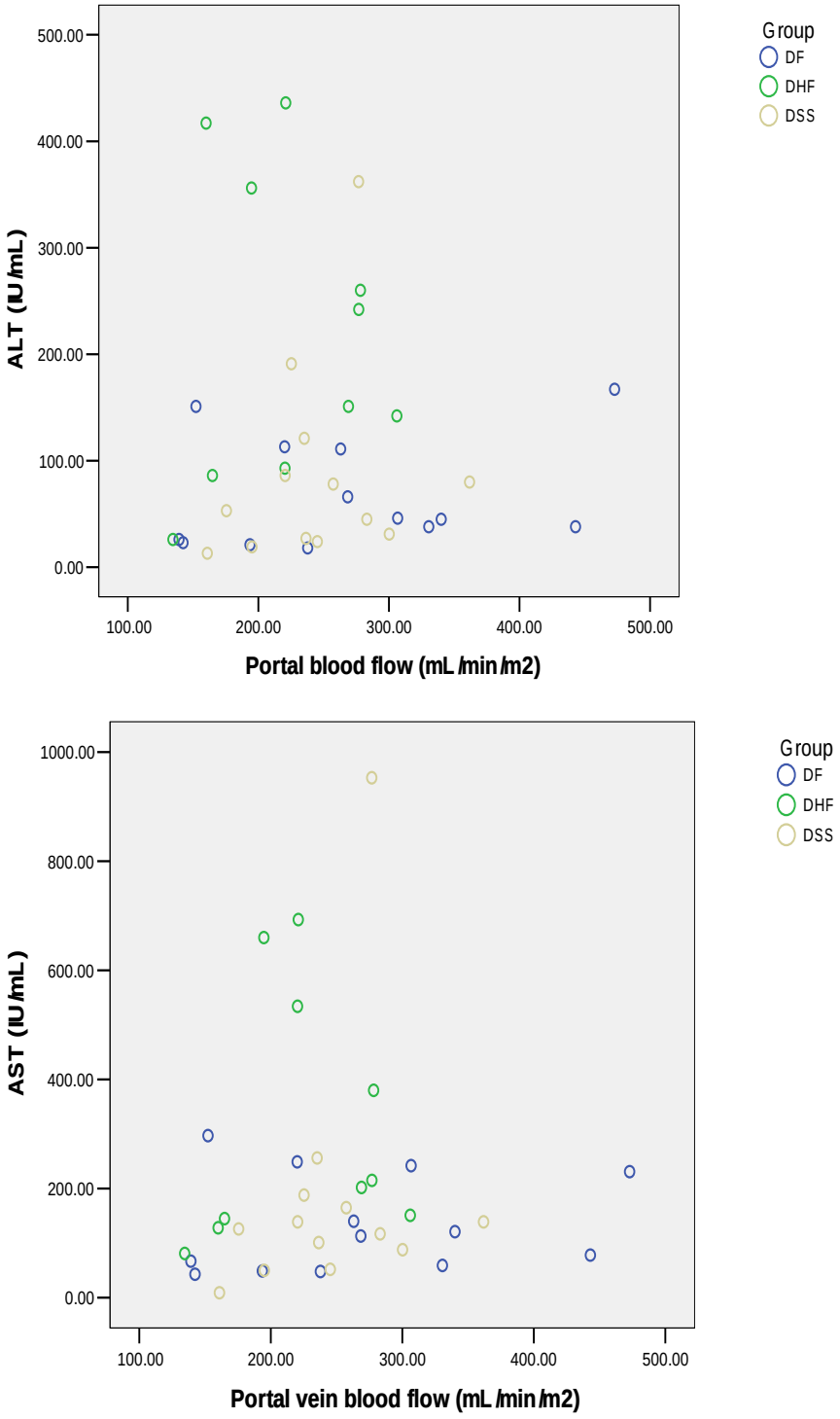
The results are summarized in the abstract below:

To study capillary lactate as hemodynamic monitoring tool in patients with dengue virus infection, lactate was determined within 6 hours of defervescence and 12 hours thereafter in 27 patients (age 10.4±3.0 years). All patients with normal initial lactate (1.8±0.3 mmol/L, n=11) received intravenous fluid of less than 650 mL/m^2 in the next 12 hours and none had pleural effusion >5% at convalescence. Among patients with high initial lactate (3.0±0.5 mmol/L, n=16), 5 (31%) had pleural effusion >5%, all of whom appeared to have relatively rapid decline of lactate. The amount of fluid administration correlated to the rate of lactate decline ($r=-0.42$, $p<0.05$). We conclude that patients with dengue virus infection with normal lactate at the initial hours after defervescence generally have mild clinical presentation. In patients with high initial lactate, subsequent follow-up level has the potential to identify the patients who received under- or over-treatment and should be further studied.

4. The relationship between portal vein blood flow and the level of ALT and AST during the toxic stage are showed in Figure 1 (page 24).

From Figure 1, there appears to be some relationship between portal blood flow and liver injury as all patients with significant elevation of ALT (> 200 IU/L) had portal blood flow of less than 300 mL/min/m^2.

Figure 1: Scatterplot demonstrating the relationship between portal blood flow (X axis) and liver enzyme (Y axis: ALT, top; and AST, bottom) in patients with dengue virus infection.



Conclusions

1. Although there were marked changes in the splanchnic circulatory system in patients with DHF (especially shock cases) compared to DF during the toxic stage, these changes were minimal or not significant during the febrile stage. These results do not support the use of ultrasonographic study of the splanchnic venous system to early predict which patients would develop DHF. Rather than the original process that led to DHF, the changes of splanchnic circulatory system were likely to be the component of DHF in itself and may be the reasons for many of the clinical presentations seen in this disease, such as abdominal distension, hepatomegaly, various gastrointestinal complaints such as abdominal pain and vomiting, etc.
2. The variables obtained during the febrile stage that we found were significantly different between patients with DF and DHF were HGF, PT and possibly ALT. All these 3 variables are related to the pathology of the liver. These findings brought along further questions whether changes in the liver might play a significant role in the pathophysiologic of DHF (as oppose to DF) in patients with dengue virus infection. Apart from using these variables (such as HGF) as an early predictor for DHF, further studies were suggested to find out the role of the liver in the pathogenesis of DHF.
3. Monitoring lactate may be of value in patients with dengue virus infection as normal value obtained when the fever subsided would make it unlikely that the patient would need a large amount of intravenous fluid resuscitation or had a severe disease. These patients may be more likely to be patients with DF and may not need extensive monitoring as compared to patients with elevated blood lactate. Health care personnel with less experience may find the test useful to triage the patients, especially in rural area when there is a large outbreak and there is over-capacity of hospital bed and/or medical personnel. However, these data are preliminary and further studies are needed to confirm these findings and to define the most appropriate target for abnormal and/or acceptable lactate level and acceptable rate to lactate normalization to avoid both under- and overtreatment in this disease.
4. From Figure 1, it can be seen that all patients with significant elevation of ALT (> 200 IU/L) had portal blood flow of less than 300 mL/min/m^2. Low portal blood flow may be an important contribution for liver injury in DHF, however, not all patients with “low” portal blood flow had elevation of AST and/or ALT. This can be explained by dual sources of blood supply common to hepatic physiology. Hepatic arterial supply may prevent liver

from ischemic insult in many patients with low portal blood flow. There could also be other factors contributing to various degree of hepatic tolerance to ischemic insults among different patients, such as the degree of hepatic congestion, degree of direct viral injury, the length of hypoperfusion and adequacy of fluid resuscitation, etc.

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Table 2: The differences of various variables between patients with dengue fever (DF) and dengue hemorrhagic fever (DHF)

Variables		Unit of measurement	Cohort	N	Data			Area under ROC curve	Remarks
					DF	DHF	p		
Standard	**Hematocrit max (Any stage)**	**%**	**1**	**117**	**40.5 ± 3.7**	**46.0 ± 4.5**	**< 0.001**	**0.83**	**Cutoff >44.2 sense 62%, spec 83%**
	Lowest Platelet (Any stage)	**X 1000 mm^3**	**1**	**107**	**88.3 ± 41.2**	**38.9 ± 26.1**	**< 0.001**	**0.87**	**Cutoff <41,000 sense 65%, spec 93%**
Splanchic Circulatory Indices	Portal vein size (indexed) (Febrile stage)	mm	1	21	5.1 ± 7.7	5.5 ± 1.1	NS	(0.67)	
	Portal vein size (indexed) (Toxic stage)	mm	1	56	5.3 ± 0.9	6.2 ± 0.9	0.001	0.75	Cutoff >5.45 sense 82%, spec 65%
	Portal vein flow velocity (Febrile stage)	cm/sec	1	22	20.7 ± 3.0	20.9 ± 5.4	NS		
	Portal vein flow velocity (Toxic stage)	cm/sec	1	57	19.7 ± 4.8	13.6 ± 4.4	< 0.001	0.85	Cutoff <14.10 sense 60%, spec 95%
	PV Congestion Index (Febrile stage)	cm-sec	1	20	0.028 ± 0.012	0.039 ± 0.022	NS	(0.68)	
	PV Congestion Index (Toxic stage)	cm-sec	1	53	0.036 ± 0.016	0.076 ± 0.048	< 0.001	0.85	Cutoff >0.058 sense 66%, spec 91%
Systemic Circulatory	IVC size (indexed) (Febrile stage)	mm	1	19	7.0 ± 1.4	7.4 ± 1.8	NS		
	IVC size (indexed) (Toxic stage)	mm	1	58	7.0 ± 2.1	6.7 ± 2.2	NS		
	LV end-diastolic volume (Febrile stage)	mL/m2	1	28	50.9 ± 11.8	53.1 ± 10.3	NS		

	LV end-diastolic volume (Toxic stage)	mL/m2	1	92	55.3 ± 12.8	44.3 ±12.6	< 0.001	0.71	
	LV ejection fraction (Febrile stage)	%	1	28	56.6 ± 10.4	63.3 ± 6.5	0.05	0.69	
	LV ejection fraction (Toxic stage)	%	1	91	59.7 ± 7.4	56.3 ± 9.2	NS		
	Heart rate (febrile stage)	/min	1	28	96.0 ± 18.6	89.2 ± 7.6	NS		
	Heart rate (Toxic stage)	/min	1	91	82.9 ±14.9	89.9 ±17.8	NS		
	Cardiac index (Febrile stage)	L/min/m2	1	28	2.77 ± 1.05	2.98 ± 0.61	NS		
	Cardiac index (Toxic stage)	L/min/m2	1	91	2.71 ± 0.84	2.18 ± 0.70	0.002	0.69	
Cytokines	ICAM-1* (Febrile stage)	ng/mL	*	16	412.0 ± 79.1	404.2 ± 105.5	NS		
	ICAM-1* (Toxic stage)	ng/mL	*	26	444.1 ± 158.0	465.1 ± 154.6	NS		
	SE-Selectin* (Febrile stage)	ng/mL	*	16	71.1 ± 30.2	85.0 ± 35.3	NS		
	SE-Selectin* (Toxic stage)	ng/mL	*	26	64.1 ± 25.7	78.8 ± 39.9	NS		
	VEGF (plasma) (Febrile stage)	pg/mL	2	6	58.7 ± 58.9	39.8 ± 8.3	NS		
	VEGF (plasma) (Toxic stage)	pg/mL	2	21	60.6 ± 49.7	41.5 ± 27.2	NS		
	MMP-2 (Febrile stage)	ng/mL	2	26	306.8 ± 102.3	297.3 ± 96.5	NS		

	MMP-2 (Toxic stage)	ng/mL	2	27	Data out of range	Data out of range	N/A		
	MMP-9 (Febrile stage)	ng/mL	2	26	306.8 ± 102.3	297.3 ± 96.5	NS		
	MMP-9 (Toxic stage)	ng/mL	2	27	373.9 ± 516.9	400.6 ± 89.7	NS		
	HGF (Febrile stage)	pg/mL	2	27	1223.9 ± 516.9	1816.5 ± 827.1	0.03	0.73	Cutoff>1627 Sense 60%, spec 88%
	HGF (Toxic stage)	pg/mL	2	27	1170 ± 544.5	1355 ± 282.0	NS		
Selected Laboratory Indices	PT** (Febrile stage)	seconds	1**	28	13.5 ±0.9	15.1 ± 1.8	0.007	0.79	Cutoff >14.35 sense 60%, spec 85%
	PT** (Toxic stage)	seconds	1**	34	12.7 ±0.8	14.2 ±2.5	0.03	0.74	
	PTT** (Febrile stage)	seconds	1**	28	46.2 ±7.1	47.8 ±10.9	NS		
	PTT** (Toxic stage)	seconds	1**	34	42.4 ± 6.9	54.5 ±11.7	0.001	0.83	Cutoff >53.1 sense 61%, spec 94%
	ALT (Febrile stage)	IU/mL	1	26	34.3 ± 30.8	99.2 ± 115.2	NS (0.07)	0.74	Cutoff>93.5 sense 31%, spec 92%
	ALT (Toxic stage)	IU/mL	1	57	65.9 ±68.6	123.0 ± 123.5	0.03	0.67	
	AST (Febrile stage)	IU/mL	1	26	100.6 ± 81.5	181.8 ± 219.8	NS		
	AST (Toxic stage)	IU/mL	1	57	123.7 ± 94.6	214.4 ± 218.7	0.043	0.67	
	Albumin (Febrile stage)	g/dL	1	26	4.0 ± 0.3	4.1 ± 0.2	NS		

	Albumin (Toxic stage)	g/dL	1	57	3.8 ± 0.5	3.4 ± 0.6	0.02	0.67	
	Urinary protein/creatinine (Febrile stage)	ratio	2	N/A	N/A	N/A	N/A		
	Urinary protein/creatinine (Toxic stage)	ratio	2	29	0.33 ± 0.26	0.56 ± 0.59	NS		

DF = dengue fever, DHF = dengue hemorrhagic fever, NS = non-significant, sense = sensitivity, spec = specificity

* = data from Khongphatthanayothin A, et al. Jpn J Infect Dis 2006;59:186-8.

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เนื้อหางานวิจัย

Manuscript 1

The Role of Vascular Endothelial Growth Factor (VEGF) Leading to Vascular Leakage in Children With Dengue Virus Infection

Patcharapa Sathupan*,**, Apichai Khongphattanayothin**, Jirabat Srisai**,***, Karakade Srikaew**,***, Yong Poovorawan**. Departments of Paediatrics, Thammasat University, Patumthani, Thailand* , Chulalongkorn University, Bangkok, Thailand** and Had Yai Hospital, Songkhla, Thailand***

Address for correspondence:
Apichai Khongphatthanayothin, M.D.
Department of Paediatrics
King Chulalongkorn Memorial Hospital
1873 Rama IV Rd., Patumwan, Bangkok 10330, THAILAND
Tel: (+66-9) 206-0384; FAX : (+66-2) 256-4911, (+66-2) 714-8524
Email: apichaik@yahoo.com
Running Title: VEGF in dengue virus infection

Abstract

Increased vascular permeability is the main aetiology for hypovolemic shock and circulatory failure in dengue haemorrhagic fever (DHF). In this study, we investigated the role of vascular endothelial growth factor (VEGF) in the pathogenesis of DHF. Serum samples from 41 patients (15 dengue fever [DF], 26 DHF) with serologically confirmed dengue virus infection during febrile, toxic, convalescent stages and at follow-up were analyzed for VEGF. Plasma samples from additional 27 children (16 DF and 11 DHF) during febrile, toxic stages and at 4-weeks follow-up visit and 8 healthy controls were analyzed for VEGF. Serum and plasma VEGF levels were not elevated during febrile or toxic stages of dengue virus infection and were not different between patients with DF and DHF. We concluded that plasma leakage in patients with DHF cannot be explained by elevation of VEGF level during the toxic stage of the illness.

Key words: Dengue, Dengue haemorrhagic fever, Dengue shock syndrome, Vascular endothelial growth factor, Vascular leakage

Introduction

Dengue haemorrhagic fever is one of the most important infectious diseases in Thailand and other tropical regions of the world. Most infected patients are asymptomatic. In those who have symptoms, their presentations are classified into 3 groups; undifferentiated fever (UF), dengue fever (DF), and dengue haemorrhagic fever (DHF). Dengue shock syndrome (DSS) is a severe complication of dengue haemorrhagic fever, characterized by massive increase in vascular permeability leading to hypovolemic shock and circulatory failure. Evidences of vascular leakage are hemoconcentration, ascites, pleural effusion, hypoalbuminemia and low central venous pressure (CVP). The pathogenesis of vascular leakage in this disease is still not clearly understood. Releases of cytokines and chemokines from monocytes and macrophages which target the endothelium are believed to play a key role in the pathogenesis of vascular leakage. [1,2]

Vascular endothelial growth factor (VEGF), originally named vascular permeability factor, is a homodimeric heparin-binding glycoprotein with potent angiogenic, endothelial-cell-specific mitogenic and vascular permeability-enhancing activities. Several studies showed that VEGF played a pathogenic role in capillary hyperpermeability that characterized ovarian hyperstimulation syndrome[3-5], preeclampsia[6] ,cirrhosis with spontaneous bacterial peritonitis (SBP)[7] and postoperative capillary leak syndrome in children undergoing cardiopulmonary bypass.[8] These findings prompted us to determine the possible role of VEGF in the pathogenesis of DHF/DSS in children in addition to the 2 recent studies that looked into this issue. [9,10]

Materials and Methods

1. Subjects

Between the years 2003-2005, 41 patients aged 5-15 years who were admitted at King Chulalongkorn Memorial Hospital with suspected dengue viral infection during the febrile stage were enrolled in this study. After informed consent was given, blood samples were collected at 4 stages: febrile stage (first day of enrolment), toxic stage (the day of defervescence), convalescent stage (discharged date) and recovery stage (at follow-up, approximately 4-6 weeks after the onset of fever) for determination of serum VEGF levels. The patients' illnesses were classified as DF or DHF according to the criteria published by the World Health Organization.[1] Patients with no serologic confirmation of dengue virus infection were excluded.

To exclude the possible effect of platelets count on serum VEGF levels, plasma samples were additionally collected from other 27 patients who were admitted at Had Yai Hospital with serologically-confirmed dengue viral infection during the years 2005-2006 for determination of the plasma VEGF level at febrile stage, toxic stage and at follow-up. Plasma samples were also obtained form 8 healthy children (age 8-14 years) as the control group for plasma VEGF determination.

The study on both periods was approved by the Ethic Committee of the Faculty of Medicine, Chulalongkorn University. Written informed consent was obtained from each subject and/or appropriate guardian prior to enrolment.

Serological confirmations of dengue viral infection were done at The Arm Force Research Institute of Medical Sciences (AFRIMS, Bangkok, Thailand) by Enzyme-Linked Immuno-Sorbent Assay (ELISA).

2. Measurements of VEGF

Three millilitres of blood sample (using EDTA as anticoagulant for plasma samples) was collected and centrifuged at approximately 1000 G. The plasma/serum was then removed and stored at $\leq$ -70°C for subsequent analysis.

Plasma and serum VEGF levels were measured by solid-phase ELISA (Quantikine R&D Systems, Minneapolis, MN, USA), designed to measure VEGF165 levels in cell culture supernatant, serum and plasma.

3. Data collection and analysis

The data were analyzed using SPSS for Windows (version 14) software. Continuous variables with normal distribution were expressed as mean (standard deviation[SD]) and were compared using one-way analysis of variance (ANOVA). Categorical variables were compared by chi-square test or Fisher exact test as appropriate. Because distribution of VEGF level and platelet count did not follow normal distribution, comparisons of these variables among DF and DHF and during the course of the disease were done by non-parametric methods (Kruskal-Wallis or Mann-Whitney U tests). The *p* value of < .05 was considered significant.

Results

1. Demographic data

Forty-one patients (16 females and 25 males) were enrolled for serum VEGF determination. The mean age was 11.1(3.1) years. There were 15 children with DF and 26 with DHF (14 had DHF without shock and 12 with DSS).

Among the 27 patients who were enrolled for plasma VEGF determination, there were 8 females and 19 males. Mean age was 9.8(2.5) years. Sixteen children had DF and 11 had DHF (10 without shock and 1 with shock). Eight healthy children (mean age = 8.75(1.63) years) serve as the control group.

The demographic data of all patients are shown in Table 1. There was no significant difference in age, sex or day of fever between patients with DF and DHF, both in serum and plasma groups.

2. Difference of serum and plasma VEGF Levels between patients with DF versus DHF.

Serum levels of VEGF in patients with DF, DHF and DSS during different stages of dengue virus infection are shown in Figure 1. A trend toward lower VEGF level in patients with higher severity of dengue virus infection was observed during febrile, toxic and convalescent stages but not at follow-up.

Plasma levels of VEGF in patients with DF, DHF and control group during febrile, toxic and recovery stages of the disease are shown in Figure 2. Similar to serum VEGF, plasma VEGF levels were lower in patients with DHF compared to DF at febrile and toxic stage. The difference between plasma VEGF in patients with DHF and DF was statistically significant at toxic stage [30.21 (inter-quartile range 27.99-34.79) versus 40.61 (inter-quartile range 32.17-51.87) pg/mL in DHF and DF, respectively, p=0.019]. There was no significant difference of plasma VEGF levels between the DHF and DF patients at febrile stage or follow-up.

3. Changes of serum and plasma VEGF levels during the course of dengue virus infection

Figure 3 demonstrated serial changes of serum and plasma VEGF during each stage of dengue virus infection. There were significant changes of serum VEGF ($p<0.001$) and plasma VEGF ($p = 0.045$) during different stages of the disease. Post-hoc analysis showed that there was no difference in serum or plasma VEGF level between febrile and toxic stages, but both serum and plasma VEGF were higher during recovery (at follow-up) compared to toxic stage ($p<0.05$).

Discussion

Vascular leakage and endothelial dysfunction are widely believed to be the main pathophysiologic processes of DHF.[1,11] Up to now, there are scanty data about the role of VEGF in the pathogenesis of vascular leakage in DHF. At first, we hypothesized that VEGF might have played a role in vascular leakage in DHF/DSS as one of its properties was enhancing the vascular permeability.

The original research idea assumed that the serum VEGF levels at follow-up could be used as normal controls but the value (mean 486 pg/mL, median 352 pg/mL) appeared to be higher than normal value reported in other studies. There were several studies reported normal serum VEGF values in children. In 2004, te Loo DM, et al. reported the mean value of serum VEGF as 290(130) pg/mL in 39 normal control children (age 2.9(1.7) years),[12] and in 2005, Ootaki Y, et al. reported the mean value as 203(221.6) pg/mL in 61 normal control children (age 6.0(3.4) years) compared to those with congenital cyanotic heart disease.[13] Because of lack of normal control and the possibility of lower platelet count causing a spuriously low serum VEGF level in patients with DHF and DSS[14,15], we additionally collected plasma samples for determination of VEGF in patients with dengue virus infection and the normal control group.

In this study, serial determination of serum and plasma VEGF levels demonstrated no elevation of VEGF during febrile or toxic stages in patients with dengue virus infection and the levels in DHF patients were not higher than the level in DF patients. In contrary to our original hypothesis, both plasma and serum VEGF appeared to be lower in patients with higher severity of dengue virus infection. Our results were different from the study by Tseng, et al. that reported higher plasma VEGF levels in adult patients with DHF compared to DF and the control group.[9] In another study, Srikiatkhachorn A, et al observed a rise in the free, but not total VEGF in plasma of DHF patients along with a decline in the soluble form of VEGF receptor 2 (sVEGFR2) at the time of plasma leakage, followed by a decline in VEGF at 4-6-day follow-up with a rise of sVEGFR2. They concluded that VEGF regulated vascular permeability and its activity was controlled by binding to sVEGFR2.[10] If VEGF is indeed the cause of increased vascular permeability in dengue haemorrhagic fever, there are two possible reasons for the low plasma VEGF in our study. First, because of its small size (molecular weight 34-42 kDa, which is much smaller than 66 kDa of albumin), plasma VEGF may be spuriously low because of its leakage out of intravascular compartment. Second, because the bound form of VEGF could not be detected by ELISA, incremental binding of VEGF to its receptor during the toxic stage may result in the low plasma level.

During the follow-up period, serum VEGF levels appeared to be increased. The reason for this observation is not clear although one could suspect that VEGF might play a role in the recovery phase of dengue infection. Further study is needed to confirm this finding.

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Table1. Demographic and clinical data

	Serum group			Plasma group		
	DF	DHF	p-value	DF	DHF	p-value
Sex (M:F)	8:7	17:9	.517	9:7	10:1	.090
Age (year)	10.5(3.3)	11.5(3.1)	.393	9.5(2.3)	10.2(2.8)	.495
Duration of fever (days)	5.1(1.2)	5.0(0.8)	.928	4.0(1.1)	3.8(1.1)	.673
Maximum Hct (%)	40.2(3.9)	45.2(4.9)	.001	41(3)	46.0(4.1)	.001
Lowest platelet count ($x10^3/mm^3$) *	94.5 (IQR 72.5-101.5)	35 (IQR 29-60)	.004	82.5 (IQR 59.5-102.8)	59 (IQR 32-73)	.110

DF = dengue fever, DHF = dengue haemorrhagic fever, Hct = Haematocrit, IQR = inter-quartile range.

* Data were presented as median (inter-quartile range)

Figure 1. The difference of serum VEGF levels between groups (DF, DHF, DSS) during each stage of dengue virus infection (the horizontal lines represent the median VEGF value for each group).

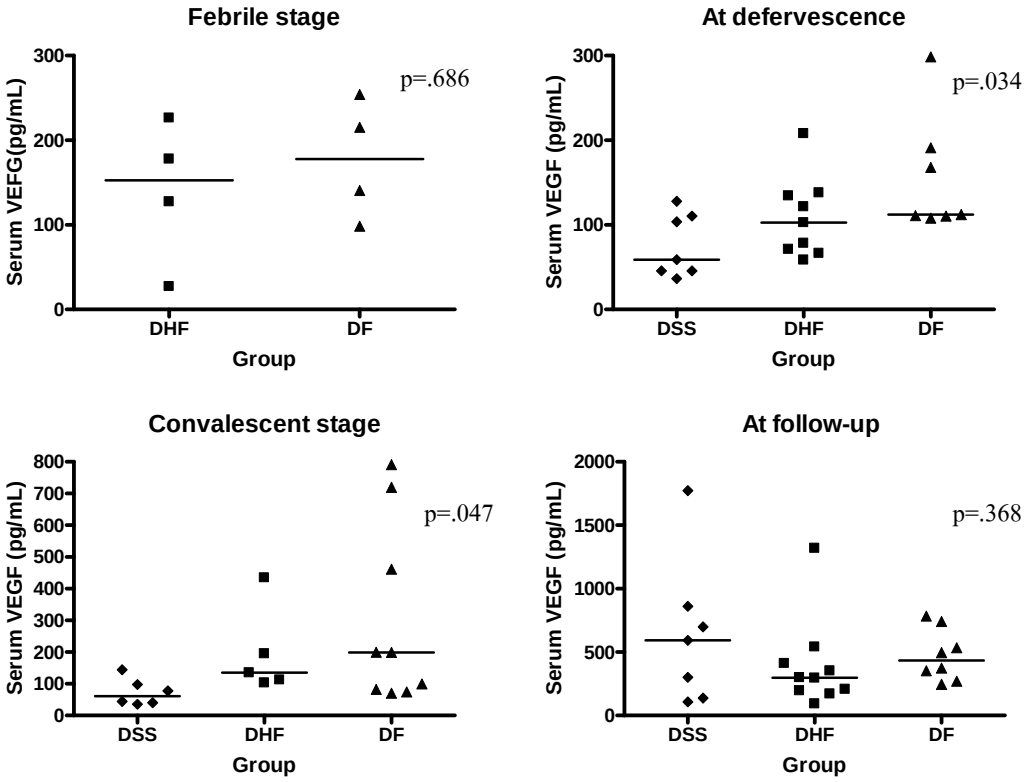


Figure 2. The difference of plasma VEGF levels between groups (DF, DHF, Control) during each stage of dengue virus infection (the horizontal line represents the median VEGF value).

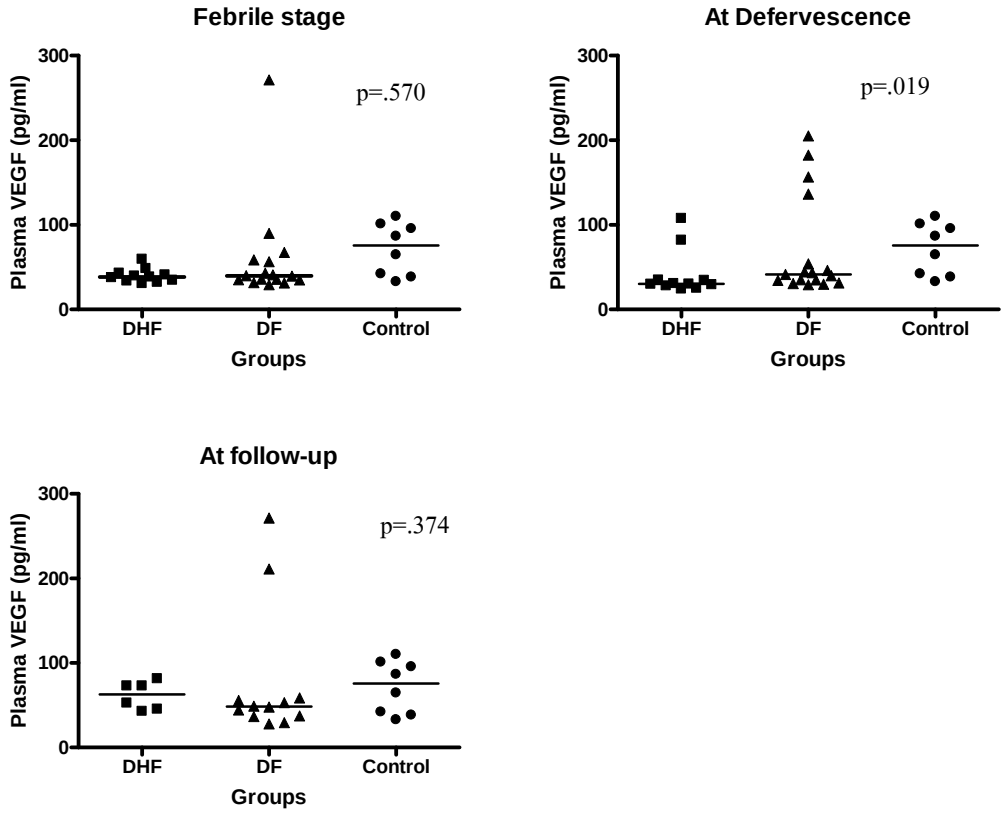
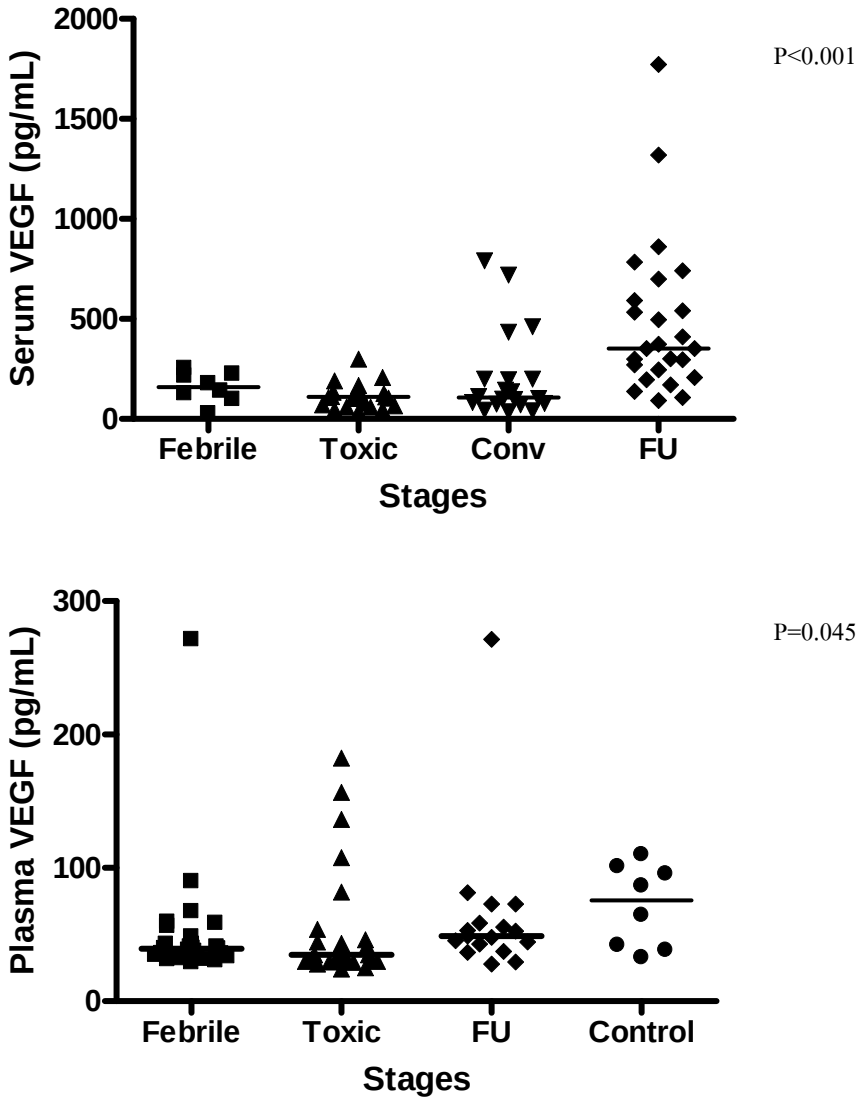


Figure 3. Serum VEGF (top) and plasma VEGF (bottom) levels during the course of dengue virus infection. The horizontal lines represent the median value for each group.



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Manuscript 2

Matrix Metalloproteinase-9 (MMP-9) in Children with Dengue Virus Infection

Nipasiri Voraphani[1], Apichai Khongphattanayothin[1], Karakade Srikaew[1,2], Praewpilai Tontulawat[1], Yong Poovorawan[1]*

Departments of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand[1], and Hadyai Hospital, Songkhla, Thailand[2]

Running head: Matrix Metalloproteinase-9 in Dengue virus infection

Keyword: Matrix Metalloproteinase-9, Children, Dengue Virus Infection

Summary

The purpose of this study was to investigate the role of MMP-9 in the pathogenesis of vascular leakage in patients with dengue virus infection. Serum samples from 24 children with serologically confirmed dengue virus infection [dengue fever (DF) = 16, dengue hemorrhagic fever (DHF) = 8, age 9.5± 2.4 years, 67% male] during the febrile, toxic stages, and at follow-up were analyzed for MMP-9. Serum samples obtained from 7 healthy children served as the control group. In patients with dengue virus infection, serum MMP-9 was lower at the febrile (227.0 ± 186.9 ng/ml) and toxic stages (150.9 ± 151.7) compared to at follow-up (424.5 ± 227.8 ng/ml) and the control group (393.3 ± 125.9 ng/ml, $p < 0.001$ by one-way ANOVA). There was no significant difference between MMP-9 levels in patients with DHF and those with DF at all stages of the disease. In conclusion, vascular leakage in patients with DHF cannot be explained by elevation of MMP-9 level.

Key Words: Dengue, Dengue hemorrhagic fever, MMP-1

Dengue hemorrhagic fever (DHF) is one of the most important emerging infectious diseases in Thailand and the world (1, 2). Plasma leakage, evidenced by hemoconcentration, ascites, or pleural effusion, is the major pathophysiological hallmark that determines disease severity and distinguishes DHF from dengue fever (DF) (3). Matrix metalloproteinase-9 (MMP-9) is an endopeptidase, which is involved in the degradation of extracellular matrix, tissue remodeling, endothelial injury, and angiogenesis (4). MMP-9 has been demonstrated to be implicated in the development of several vascular conditions and diseases (5-7). However, its significance in patients with dengue virus infection has never been studied. We hypothesized that MMP-9 may play a crucial role in the mechanism of plasma leakage in DHF by causing injury to extracellular matrix, resulting in endothelial cell swelling and detachment from the basement membrane. The objective of this study was to determine the level of MMP-9 in children with dengue virus infection.

Blood samples at the febrile, toxic stages, and at follow-up (FU, at least 1 week after defervescence) in 24 patients (age 9.5 ± 2.4 years, male/female = 18/8, DF = 16 and DHF = 8) were collected. Serum MMP-9 level was determined by a commercially available ELISA kit (Quantikine R&D Systems, Minneapolis, MN, USA). All patients had serological or polymerase chain reaction confirmation of dengue virus infection. Diagnosis and grading of DHF was done according to the criteria published by the World Health Organization (WHO) (3). The research protocol was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University. Written informed consent was obtained from an appropriate guardian and/or the patient prior to enrollment.

A total of 61 serum samples were analyzed for MMP-9 levels. Seven healthy children served as normal controls. Demographic and clinical data of all subjects are summarized in Table 1.

Table 1: Demographic and clinical data of 24 patients enrolled.

	DF (n = 16)	DHF (n = 8)	*p*-value
Age (years)	9.4 ± 2.3	9.8 ± 2.6	NS
Sex (M/F)	8/8	8/0	<0.05
Body temperature max (^{o}C)	39.6 ± 1.1	39.7 ± 0.5	NS
Maximal Hematocrit (%)	40.4 ± 3.1	46.3 ± 3.4	< 0.01
Lowest Platelet (x10^{3}/mm^{3})	88 ± 40	81 ± 76	NS

DF = dengue fever, DHF = dengue hemorrhagic fever, NS = not significant

Serum MMP-9 levels in patients with dengue virus infection (DF+DHF) during different stages of the disease and in controls are shown in Figure 1. Serum MMP-9 was significantly

different when comparing between the 3 stages of the disease (p<0.001 by one-way of variance). Post-hoc analysis showed that the MMP-9 level was significantly lower in patients with dengue virus infection at the febrile and toxic stages compared to at follow-up (FU) and the level in the control group (227.0 ± 186.9 for febrile stage and 150.9 ± 151.7 for toxic stage vs. 424.5 ± 227.8 ng/ml for FU and 393.3 ± 125.9 ng/ml for the control group, $p < 0.05$ for febrile vs. FU, toxic vs. FU and toxic vs. control). Figure 2 shows the difference between MMP-9 levels in patients with DF and DHF at different stages of the disease. Serum MMP-9 appeared to be lower in DHF patients compared to DF patients but these results were not statistically significant.

Recent in-vitro and mouse experiments have revealed the importance of MMPs in dengue virus infection-induced vascular leakage. Luplerdlop N, et al found that dengue virus-infected immature dendritic cells overproduced MMP-9 in a virus-dose dependent manner (8). The increase of this protein led to enhancement of vascular permeability, which could be reduced by specific inhibitors. Moreover, the elevated level of tissue inhibitors of metalloproteinases (TIMP)-1, the natural inhibitor of MMP-9, was demonstrated (8). In another study, dengue virus infection of primary human endothelial cells powerfully increased MMP-2 production, and to a lesser extent, of MMP-9 (9). Overproduction of MMP-9 was thought to be the potential explanation of endothelial injury and vascular leakage in children with DHF.

Contrary to the original hypothesis, our study demonstrated that MMP-9 level was not elevated during febrile or toxic stages of dengue virus infection. These data do not support the role of MMP-9 in the pathogenesis of the illness caused by dengue virus. The cause of lower MMP-9 in patients with dengue virus infection at the toxic stage, especially in DHF, is unclear. There are two possible reasons to explain this finding. First, there may be a rapid increase in the inhibitors of MMPs, such as TIMPs and α_2-macroglobulin in serum of dengue-infected patients as an attempt of the body to restore the physiological process as shown in the in-vitro study. Second, with its molecular weight just slightly higher than albumin (92 kDa compared to 66 kDa), MMP-9 level may be lower during the toxic stage because of its leakage into the interstitial space. Further studies are warranted to explain the cause of lower MMP-9 in-vivo and the potential role of its inhibitors in the pathophysiologic process of dengue virus infection.

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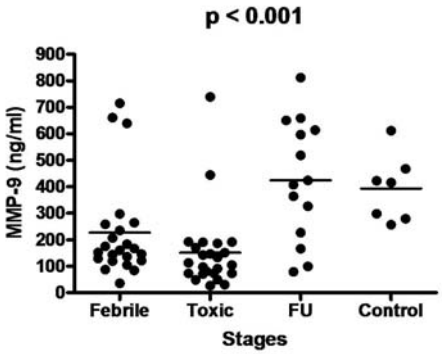

Figure 1: MMP-9 level in children with dengue infection during different stages of the illness (p<0.001 by one-way analysis of varience). FU = follow-up.

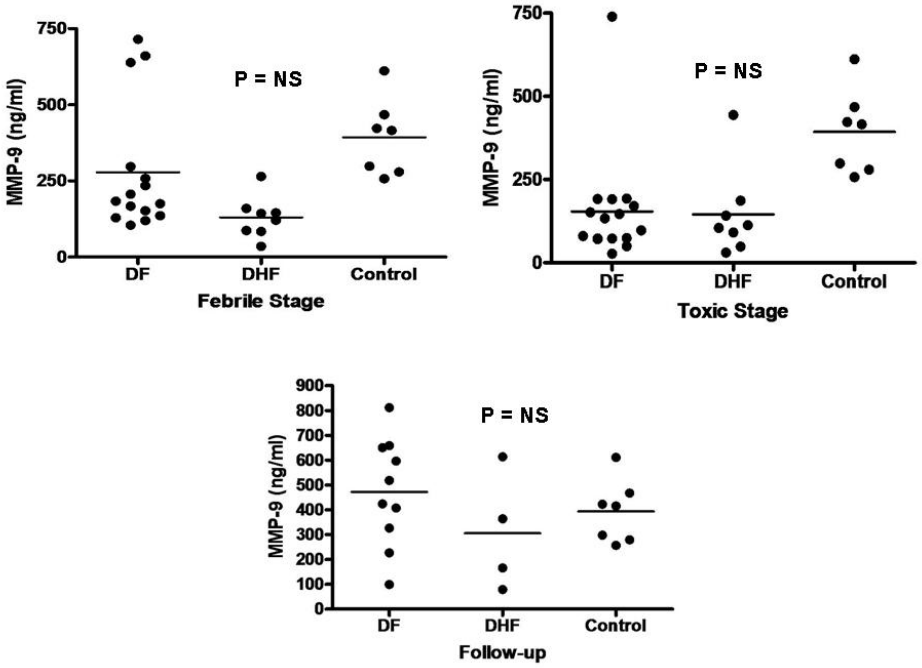

Figure 2: MMP-9 levels in patients with dengue fever (DF) vs dengue hemorrhagic fever (DHF) at different stages of the illness. NS = not significant.

Manuscript 3

Increased Level of Hepatocyte Growth Factor in Children with Dengue Virus Infection

NIPASIRI VORAPHANI[1], APIRADEE THEAMBOONLERS[1], APICHAI KHONGPHATTHANAYOTHIN[1], CHIRABAT SRISAI[1,2], YONG POOVORAWAN[1*]

Department of Paediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand[1], and Hadyai Hospital, Songkhla, Thailand[2]

Running head: Hepatocyte growth factor in dengue virus infection

Keyword: Hepatocyte growth factor, Children, Dengue, Dengue haemorrhagic fever

ABSTRACT

Background: Evidence of hepatocellular damage is common in dengue-infected individuals. Hepatocyte growth factor (HGF), a key cytokine responsible for liver regeneration, may play a prognostic role in dengue virus infection.

Aim: To determine the relationship between serum HGF level and disease severity in patients with dengue virus infection.

Methods: Serum samples from 27 children [17 dengue fever (DF), 10 dengue haemorrhagic fever (DHF)] with serologically confirmed dengue virus infection during the febrile, toxic stages, and at follow-up were analysed for HGF. Serum samples obtained from 9 healthy children served as the control group.

Results: In dengue-infected patients, serum HGF was significantly higher at the febrile and toxic stages than at follow-up ($p<0.05$). In comparison with DF, patients with DHF had a greater level of HGF at the febrile stage ($p<0.05$). A cut-off HGF level of 1,220 pg/mL obtained during the febrile stage showed a sensitivity of 90%, and a specificity of 53%, for predicting the clinical progression to DHF (area under the ROC curve = 0.75).

Conclusion: Serum HGF level at the early stage of dengue virus infection is elevated and may be a useful predictor for clinical progression to DHF.

Introduction

Dengue virus infection is emerging as a major public health problem, principally in tropical countries, and expanding globally with an estimated annual incidence of 50 to 100 million cases. The clinical spectrum of dengue infection ranges from asymptomatic to dengue fever (DF), dengue haemorrhagic fever (DHF), and a severe, potentially fatal form called dengue shock syndrome (DSS).[1] The liver is one of the major target organs of dengue virus and hepatocellular damage can occur in varying degrees during the course of the disease.[2, 3]

Hepatic dysfunction is common in dengue-infected individuals, including painful hepatomegaly and increased levels of the hepatic enzymes, aspartate transaminase (AST) and alanine transaminase (ALT). The elevation of liver enzymes, predominantly AST, is significantly greater in patients with DHF and DSS than DF, suggesting that the magnitude of liver injury is associated with disease severity.[2] Generally, the increase in transaminase level is mild to moderate, reaching maximum values around the ninth day after the onset of fever and gradually normalising thereafter within a few weeks.[3] Although the extent of liver involvement in patients with dengue virus infection is usually mild, fulminant hepatitis and liver failure as further complications of severe DHF/DSS have also been described, mainly in children and young adults, and are associated with a poor prognosis.[4, 5]

The exact pathogenic mechanism of hepatocellular injury during dengue virus infection is not fully understood. It could be the direct effect of virus on liver cells or the result of a dysregulated immune response to the virus.[6] Histopathological findings of fatal cases revealed centrilobular necrosis, microvesicular steatosis, Kupffer cell hyperplasia, mononuclear cell infiltration in the portal tract, and Councilman bodies, indicating an apoptotic process.[7, 8] A range of proinflammatory, and immunoregulatory cytokines as well as various chemokines have been studied in the pathogenesis of dengue virus infection.

Hepatocyte growth factor (HGF) is a pleiotropic cytokine, which has been recognised as the most potent mitogen for mature hepatocytes. This cytokine plays an essential role in liver regeneration after injury.[9] Increase in serum HGF has been observed in various liver diseases, such as alcoholic hepatitis, fulminant hepatic failure, and hepatocellular carcinoma.[10-13] In addition, it may serve as a predictor for disease severity and outcome. Although hepatic damage in dengue infection has been frequently described, the role of HGF in this illness has never been studied. Hence, this project was designed to determine the relationship between serum HGF level and disease severity in children hospitalised with dengue infection in Thailand.

Materials and Methods

Study patients

From January 2005 to December 2006, children admitted to Had Yai Hospital, Songkhla, with the initial diagnosis of dengue virus infection were enrolled. The patient was included if he/she fulfilled all of the following criteria: 1) age 5-15 years and 2), had serological confirmation of dengue virus infection. Exclusion criteria were: 1) any other acute/chronic diseases previously diagnosed or 2) refusal of consent to the study by patient and/or parents. The research protocol was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University. Written informed consent was obtained from an appropriate guardian and/or the patient prior to enrolment.

Blood sample collection

Upon having obtained informed consent, blood samples were collected at 3 stages: febrile stage (first day of enrolment), toxic stage (the day of defervescence), and recovery stage (at follow-up) for determination of serum HGF level. Three millilitres of blood sample were collected and centrifuged at approximately 1000 G. The serum was then removed and stored at $\leq$ -70°C for subsequent analysis.

Determination of HGF level

HGF level was determined by a commercially available ELISA kit (Quantikine R&D Systems, Minneapolis, MN, USA).

Determination of right pleural effusion

The amount of right pleural effusion was determined by ultrasonographic examination of the right pleural space. A transducer was placed at the right postero-lateral aspect of the chest wall, at the level of the xiphoid process. The maximum width of the pleural effusion was measured. Any pleural effusion would qualify the patient for diagnosis of DHF.

Clinical data

The progression of the patient's illness was classified into three clinical stages as febrile, toxic and convalescent stages according to the World Health Organization (WHO) criteria.[1] Toxic stage was defined as the day of defervescence or presence of haemoconcentration and/or shock. Convalescent stage was defined as 24-48 hours after the toxic stage and once the patient was recovering. Follow up was performed at least 1 week after

the toxic stage. Acute and convalescent sera from all subjects were sent for serological confirmation of dengue virus. Dengue virus infection was diagnosed and classified as DF, DHF and DSS according to the WHO case definition.[1] Diagnosis of DHF required at least 1 evidence of capillary leakage (rising of haematocrit > 20% or presence of pleural effusion and/or ascites). In patients without baseline haematocrit, the percentage of haemoconcentration was determined using the difference between maximal and minimal haematocrit during the same admission. DSS was diagnosed when the patient with DHF had clinical findings of shock such as: 1) hypotension in relation to age and cold clammy skin or restlessness or 2) narrow pulse pressure (< 20 mmHg.) and rapid and weak pulse.[1] The classification of the patient's clinical severity was agreed upon by two physicians. All patients were treated by our in-house staff and attending staff according to the guideline published by the WHO.[1]

Confirmation of dengue virus infection

Serologic studies of dengue virus infection were performed at the Department of Medical Sciences, Ministry of Public Health (Nonthaburi, Thailand) using enzyme-linked immunosorbent assay (ELISA).

Data collection and analysis

The data were analysed using Prism for Windows version 5 (GraphPad Software Inc.,La Jolla, CA, USA). Continuous variables were expressed as mean ± standard deviation and were compared using t-test or one-way ANOVA. Categorical variables were compared by chi-square test or Fisher exact test as appropriate. A *p*-value of < .05 was considered significant.

Results

Sixty-nine serum samples from 27 patients with serologically-confirmed dengue virus infection (17 with DF and 10 with DHF) were analysed for serum HGF levels. Nine healthy children served as normal controls. Demographic and clinical data of all subjects are summarised in Table 1.

HGF serum levels in patients with dengue virus infection (DF+DHF) during different stages of the disease and in controls are shown in Figure 1. Serum HGF was significantly different when comparing between the 3 stages of the disease (p<0.001). Post-hoc analysis showed that the HGF level was significantly higher in dengue-infected patients at the febrile and toxic stages than at follow-up (p<0.05).

Figure 2 shows the difference between HGF levels in patients with DF and DHF at different stages of the disease. Serum HGF was significantly different when comparing between DF and DHF patients at the febrile stage (top figure, p<0.05). There was a tendency towards a higher level of serum HGF in patients with DHF than those with DF at the toxic stage (centre figure, p=0.33), but not at follow-up (bottom figure, p=0.96). Receiver operating characteristics analysis showed that the area under the curve was 0.75 for utilising the HGF level at the febrile stage for predicting clinical progression to DHF (Figure 3). A cut-off serum HGF level of 1,220 pg/mL obtained during the febrile stage showed a sensitivity of 90%, and specificity of 53%, for predicting progression to DHF.

The ultrasonographic examination of the right pleural space was performed in 23 patients at the convalescent stage. Five children were found to have pleural effusion. There was no significant relationship between pleural effusion thickness and serum HGF level at the febrile and toxic stages (r = 0.26, p= 0.22 for febrile and r = 0.02, p=0.95 for toxic stage, by linear regression analyses).

Discussion

HGF is a heparin-binding polypeptide growth factor which is known to be the most potent trigger of hepatocyte regeneration following liver injury. This cytokine is synthesised by a range of cells including non-parenchymal liver cells, such as sinusoidal wall endothelial cells, Kupffer cells, and Ito cells.[15] Various studies conducted on patients with various types of infectious and non-infectious liver diseases including hepatitis B, fulminant hepatic failure, and hepatocellular carcinoma, have demonstrated a variable degree of increased serum HGF levels.[12, 13, 16] Some research has also investigated its correlation with biochemical parameters of hepatic damage and supported its value as a prognostic marker.

Evidence of hepatocellular damage in dengue-infected patients, as indicated by raised transaminase levels (predominantly AST) has been described and was more pronounced in DHF than DF.[2] Nevertheless, the level of HGF during dengue infection has never been investigated. We initially hypothesised that elevated serum HGF levels might be associated with dengue virus infection and related to disease severity.

In accordance with the original hypothesis, our study demonstrated that serum HGF levels were significantly elevated during the febrile and toxic stages of dengue infection. Furthermore, DHF patients tended to have a higher HGF level than DF patients during the febrile stage (Figure 2, top). Although HGF appeared to be higher in DHF patients compared to DF patients at the toxic stage (Figure 2, centre), the difference did not reach statistical significance possibly due to the relatively small sample size. The early increase in serum HGF level observed in our study might have a prognostic value in a patient suspected to have dengue virus infection. A cut-off level of 1,220 pg/mL obtained during the febrile stage was found to be a sensitive predictor for clinical progression to DHF (sensitivity 90%). Therefore, serum HGF obtained at the febrile stage might become a valuable tool for early identification of dengue-infected patients at risk for developing DHF and should be further studied. Due to small sample sizes, the relationship between HGF and severity of pleural effusion was not statistically significant. Additional studies with more subjects are required to better elucidate these findings.

The mechanism underlying increased HGF level in patients with dengue infection, particularly DHF, has remained unclear. We postulate that it might be due to enhanced production of this hepatotropic factor in response to dengue-induced liver injury, which has previously been reported to be more profound in DHF than in DF.[2] In addition, since the liver is responsible for clearance of HFG, decreased elimination as a result of liver cell damage could be another possible explanation. Additional investigations are required to elucidate the possible mechanisms and the significance of HGF elevation in dengue infection.

In summary, we provided preliminary data suggesting that serum HGF level at the early stage of dengue virus infection is elevated and may serve as a useful predictor for clinical progression to DHF. Further studies with larger patient groups are warranted to confirm these encouraging results.

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Figure Legend

Figure 1: HGF level in children with dengue infection during different stages of the illness (p<0.001).

Figure 2: HGF level in patients with DF vs. DHF at different stages of the illness (p<0.05 at the febrile stage).

Figure 3: Receiver operative characteristic (ROC) curve for using HGF during febrile stage to predict clinical progression to DHF versus DF (area under the curve = 0.75).

Table 1: Demographic and clinical data of 27 patients enrolled.

	DF (n = 17)	DHF (n = 10)	*p*-value
Age (years)	9.7 ± 2.4	9.9 ± 2.8	NS
Sex (M/F)	9/8	9/1	NS
Body temperature max (C)	39.6 ± 1.0	39.7 ± 0.5	NS
Maximal Haematocrit (%)	41.0 ± 3.9	45.5 ± 4.0	< 0.01
Lowest Platelet ($x10^9$/L)	86 ± 40	74 ± 70	NS

NS = not significant

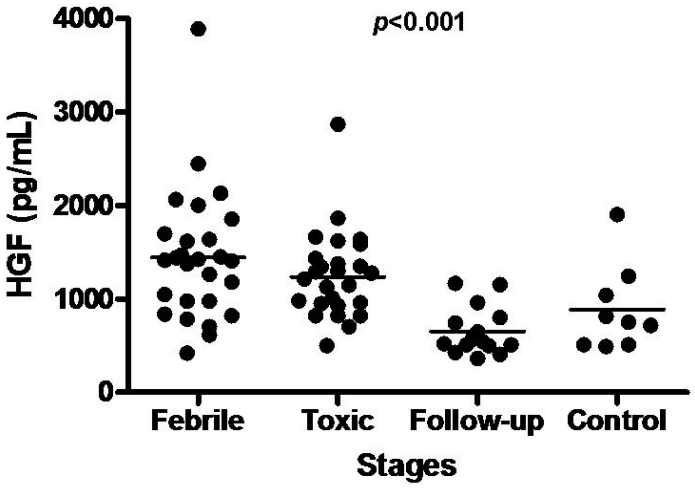

Figure 1: HGF level in children with dengue infection during different stages of the illness (p<0.001).

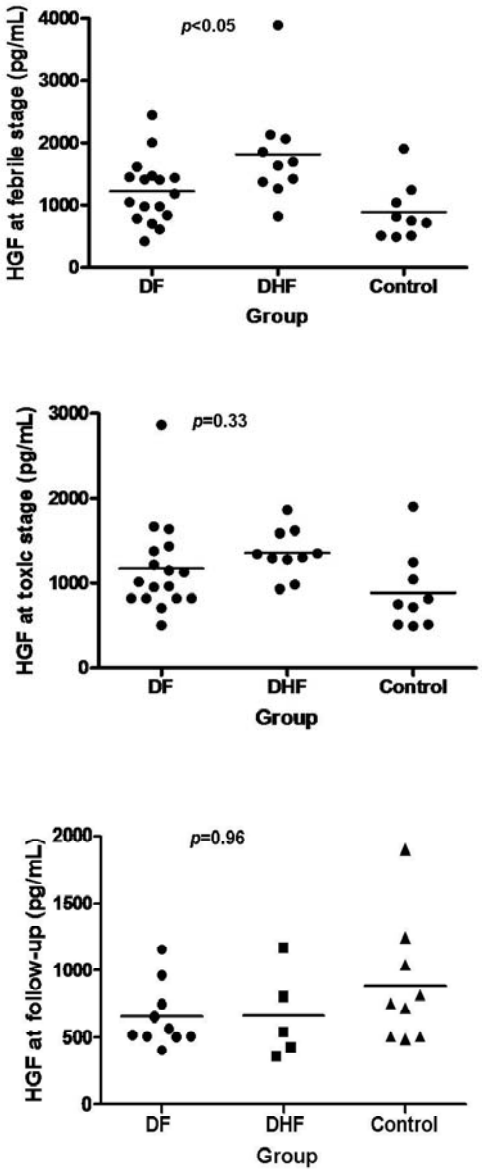

Figure 2: HGF level in patients with DF vs. DHF at different stages of the illness (p<0.05 at the febrile stage).

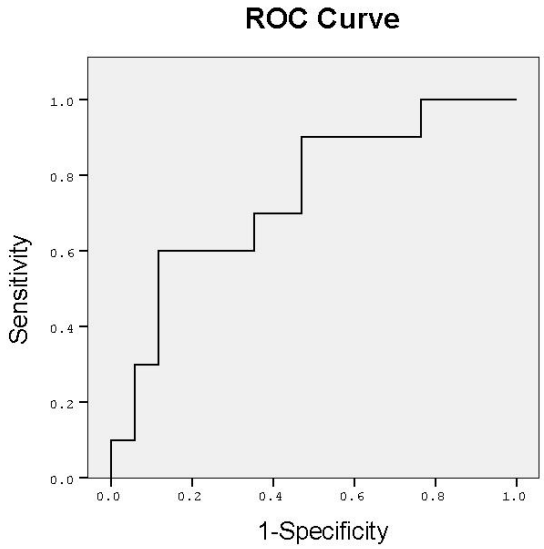

Figure 3: Receiver operative characteristic (ROC) curve for using HGF during febrile stage to predict clinical progression to DHF versus DF (area under the curve = 0.75).

Manuscript 4

Capillary Lactate as a Potential Hemodynamic Monitoring Tool in Patients with Dengue Virus Infection

Nawarat Rungteeranon*, Pentip Supachokechaiwattana**, Kanyalak Vithessonthi*, Vidhavas La-orkhun*, Pornthep Lertsapcharoen*, Apichai Khongphatthanayothin*.
Departments of Paediatrics, Faculty of Medicine, Chulalongkorn University* and Sawanpracharak Hospital**, Bangkok and Nakorn Sawan, Thailand.

Summary

To study capillary lactate as hemodynamic monitoring tool in patients with dengue virus infection, lactate was determined within 6 hours of defervescence and 12 hours thereafter in 27 patients (age 10.4±3.0 years). All patients with normal initial lactate (1.8±0.3 mmol/L, n=11) received intravenous fluid of less than 650 mL/m^2 in the next 12 hours and none had pleural effusion >5% at convalescence. Among patients with high initial lactate (3.0±0.5 mmol/L, n=16), 5 (31%) had pleural effusion >5%, all of whom appeared to have relatively rapid decline of lactate. The amount of fluid administration correlated to the rate of lactate decline ($r=-0.42$, $p<0.05$). We conclude that patients with dengue virus infection with normal lactate at the initial hours after defervescence generally have mild clinical presentation. In patients with high initial lactate, subsequent follow-up level has the potential to identify the patients who received under- or over-treatment and should be further studied.

Key words: Dengue, Dengue hemorrhagic fever, Lactate, Hemodynamic monitoring, Children

Low cardiac output and shock are one of the most important consequences of dengue virus (DV) infection[1,2]. Lactate elevation in bacterial sepsis and/or septic shock has been shown to correlate with the disease severity and outcome and is frequently monitored in these patients[3,4]. The purpose of this study was to evaluate the potential role of blood lactate as a hemodynamic monitoring tool in patients with DV infection.

Materials and Methods:

Subjects: Children (age 0-15 years) with ELISA or PCR-proven DV infection who were admitted to King Chulalongkorn Memorial Hospital (Bangkok, Thailand) or Sawanpracharak Hospital (Nakorn Sawan,Thailand) were enrolled. We excluded patients with underlying heart or metabolic diseases which may affect blood lactate level.

Lactate determination: Capillary blood was used for lactate determination by a hand-held device (Accutrend Lactate, Roach Diagnostics, Germany) within 6 hours after defervescence (toxic stage) and 12 hours thereafter. Lactate level of $\leq$ 2.2 mmol/L was considered to be normal[5].

Determination of pleural effusion:

The amount of right pleural effusion during the convalescent stage was determined by ultrasonographic examination of the right pleural space as previously described[6]. Pleural effusion index (PEI) was calculated as the width of right pleural effusion divided by the width of the patient's hemithorax at level of the xiphoid process.

Clinical data:

The progression of the patient's illness was classified into three clinical stages as febrile, toxic and convalescent stages and as dengue fever (DF), dengue hemorrhagic fever without shock (DHF) and dengue shock syndrome (DSS) according to the criteria published by the World Health Organization (WHO)[1]. All patients were treated by our house staff and/or attending staff according to the WHO guideline. Lactate levels were not known to the physician who treated the patient.

Variables and Statistics:

Data are expressed as mean ± SD. Unpaired t-test or one-way ANOVA were used to compare continuous variables and Chi-square test was used to compare categorical variables among 2 or more groups. Correlation between 2 continuous variables was assessed by linear regression.

Ethic committee approval and patient's consent

The study was approved by the Ethic Committee of the Faculty of Medicine, Chulalongkorn University and Sawanpracharak Hospital. Written informed consent was obtained from each subject and/or appropriate guardian prior to enrollment.

Results

Twenty-seven patients were enrolled. The demographic and clinical data of all patients are shown in Table 1. Twelve patients were classified as DF, 11 as DHF and 4 as DSS. All patients were successfully treated by intravenous fluid alone (no inotropic agent) and no mortality occurred in our cohort.

The levels of capillary lactate at different stages of the illness are shown in Figure 1. Elevated lactate (> 2.2 mmoL/L) was found in 59 and 61% of the first and second determinations during the toxic stage, respectively.

Correlation between blood lactate and the clinical course and outcome.

The initial capillary lactate during the toxic stage in this cohort ranged from 1.2 to 3.7 mmol/L. Lactate levels determined during the early toxic stage (first determination) were significantly different among patients with DF (2.2±0.6 mmol/L), DHF (2.6±0.6 mmol/L) and DSS (3.2±0.6 mmol/L, n = 4), respectively (p=0.03).

Using 2.2 mmol/L as the highest limit for normal lactate value[5], 11 and 16 patients were found to have normal and elevated capillary lactate on the first determination during the toxic stage, respectively. Figure 2 demonstrates the amount of intravenous fluid administration in the next 12 hours (top figure), and pleural effusion index (PEI) during convalescent stage (bottom figure) in patients with normal lactate (1.8±0.3 mmol/L, n = 11, left column) and high lactate (3.0±0.5 mmol/L, n = 16, right column). All patients with normal initial lactate received intravenous fluid of less than 650 mL/m^2 in the next 12 hours and none had PEI of > 5% at convalescence. The amount of fluid administration and PEI in patients with elevated lactate level varied considerably (right-handed column), with some patients received large amount of

fluid resuscitation and/or developed large amount of pleural effusion.

The relationship between lactate change and fluid administration and pleural effusion

Figure 3 demonstrates the relationship between the amount of intravenous fluid administration (top figure), and pleural effusion index (PEI, bottom figure) versus the changes of blood lactate. In general, lactate level declined more rapidly in patients with larger amount of intravenous fluid administration (top figure; $r = -0.42$, $p < 0.05$). The amount of pleural effusion appeared to be larger in patients with larger drop of lactate level in the first 12 hours of toxic stage as well. The correlation coefficient of the association was however, not statistically significant (bottom figure; $r = -0.26$, $p = 0.22$).

Five patients in our cohort had PEI at convalescent stage of > 5%. All of these patients belonged to the group with high initial lactate during the toxic stage (Figure 2). Compared with the other patients in this group, these 5 patients appeared to receive larger amount of intravenous fluid in the 12-hour interval between the 2 lactate determination (981±516 vs. 658±460 mL/m^2, $p = 0.24$) and had larger decline in blood lactate (-0.5±0.3 mmol/L vs. +0.1±0.8 mmoL/L, $p = 0.14$) but the differences did not reach statistical significance.

Discussion

Lactate determination has been evaluated and used for hemodynamic monitoring in many diseases that are associated with low cardiac output and/or shock[3-5]. The potential value of lactate determination in patients with DV infection has not been previously studied and is the focus of this report.

In this study, we found that initial lactate determination within 6 hours of defervescence correlated with the subsequent treatment and outcome. Normal lactate at this stage was associated with a mild disease. The clinical progression of patients with elevated initial lactate was more variable with some patients receiving more intravenous fluid and/or developing larger pleural effusion compared to the others. As expected, we found that larger amount of fluid administration generally resulted in more rapid lactate decline, the finding that would be cheered in patients with bacterial sepsis. In patients with DV infection, however, faster decline of lactate may be associated with larger pleural effusion as shown in Figure 3 (bottom). Because respiratory embarrassment from pleural effusion and/or pulmonary edema can occur from over-administration of intravenous fluid, and mild hyperlactatemia did not appear to be harmful in patients with DV infection, it appeared that the rapid decline of lactate sought for in the treatment of septic shock[3,4] may not be warranted in patients with DV infection, possibly

except for those with shock or severe disease. Further study is needed to confirm these findings and to determine the value of serial lactate determination in patients with DV infection including the level that can be safely tolerated and the proper degree of lactate decline to prevent both under- and over-treatment in this disease.

Funding

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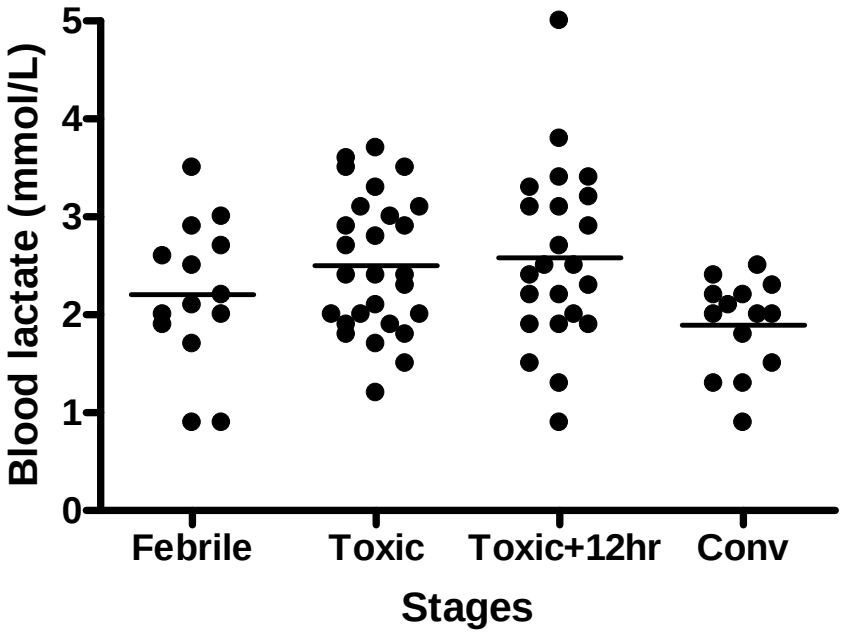
Table1: The demographic and clinical data of all patients

	DF (n = 12)	DHF (n = 11)	DSS (n = 4)	p-value
Sex (M:F)	9:3	4:7	4:0	0.04
Age (yr)	11.4±3.1	9.6±2.8	9.7±3.1	0.38
Body weight (Kg)	46.8±19.0	33.1±15.2	39.3±9.0	0.16
Duration of fever (days)	5.0±0.6	4.7±0.9	4.8±1.0	0.69
Maximum Hct (%)	41.2±2.7	45.4±3.1	45.0± 4.7	0.01
Lowest Plt ($x10^3/mm^3$)	61.8±22.9	49.3±26.7	61.3±33.4	0.49
PEI (mm)	0	7.1±9.8	7.5±10.7	0.06
Lactate level during toxic stage	2.2±0.6	2.6±0.6	3.2±0.6	0.03

DF ;dengue fever, DHF ;dengue hemorrhagic fever, Hct ;hematocrit, Plt; platelet number, PEI; pleural effusion index

Figure 1: Blood lactate during different stages of dengue virus infection



Horizontal line = mean value. Conv = convalescent stage, Toxic+12hr = 12 hours after the first level during toxic stage.

Figure 2: Amount of initial fluid administration in 12 hours (top) and pleural effusion index (PEI) at convalescent stage (bottom) based on the level of first lactate during toxic stage.

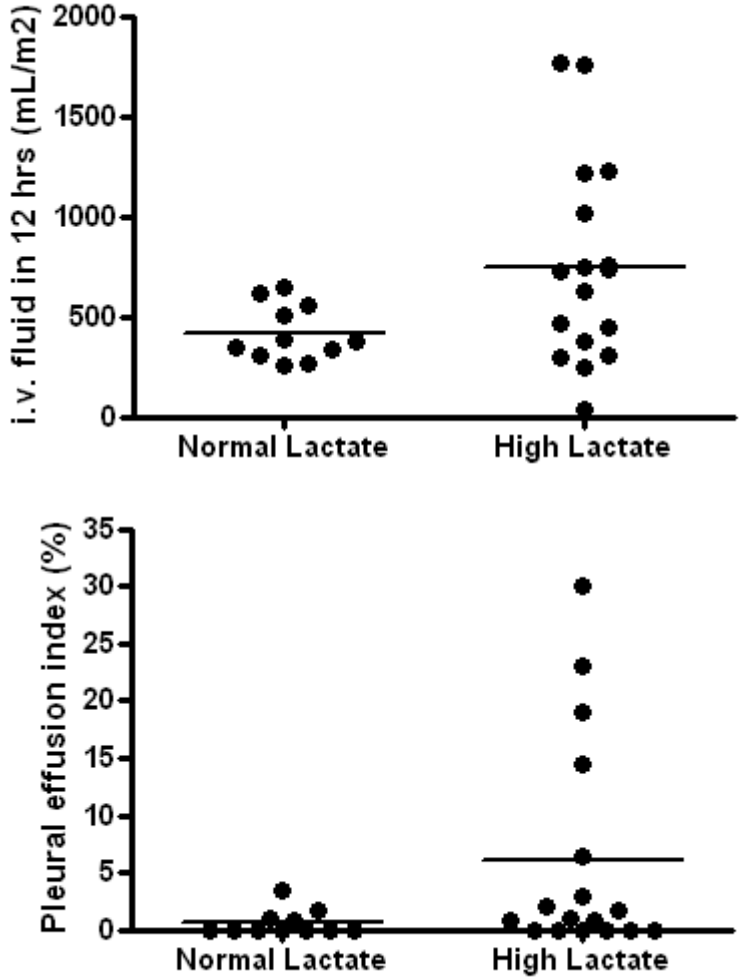
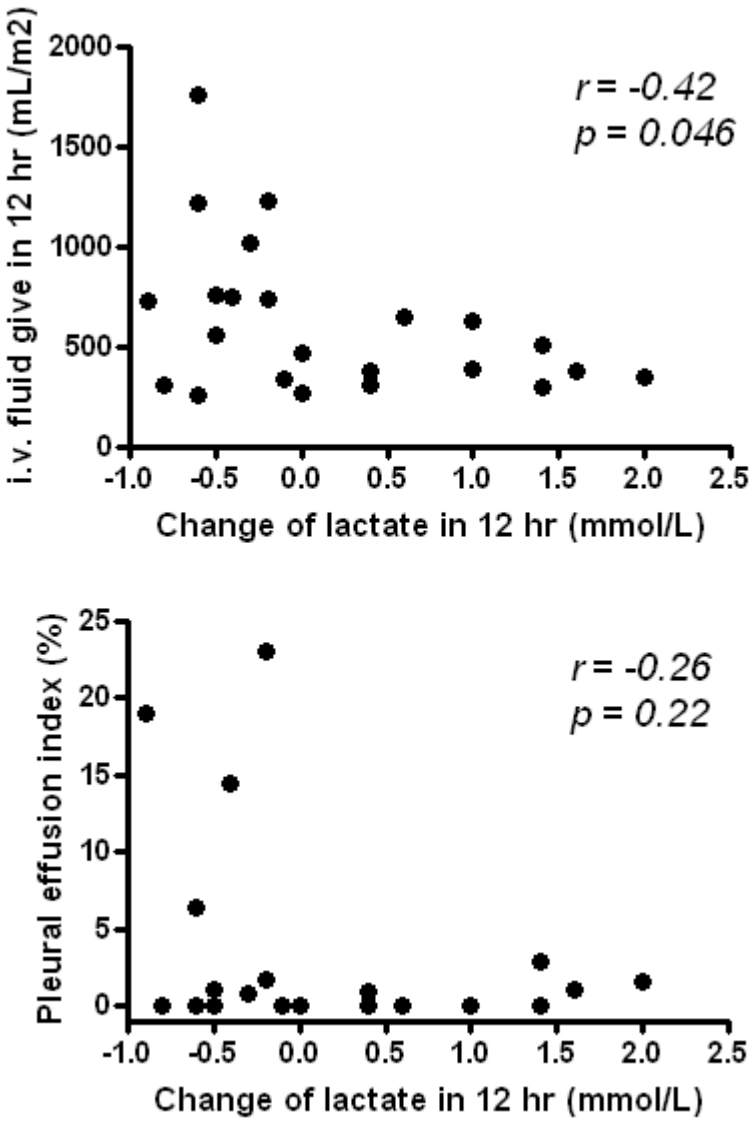


Figure 3: The relationship between the amount of fluid administration in the first 12 hours of toxic stage (Y axis, top figure) and pleural effusion index (PEI) at convalescent stage (Y axis, bottom figure) versus the change in blood lactate during the toxic stage (X axis).



Manuscript 5

Relationship between portal blood flow and liver enzyme elevation in patients with dengue virus infection: Is liver injury a result of hepatic hypoperfusion?

Apichai Khongphatthanayothin, Pasjutha Ratanasukol, Pentip Supachokechaiwattana, Kanyalak Sethkrikul, Yong Poovorawan.

Department of Pediatrics, Faculty of Medicine, Chulalongkorn University and King Chulalongkorn Memorial Hospital, Bangkok, Thailand

This study was supported by The Thailand Research Fund (Grant# RMU 4980019).

Address for correspondence:

Apichai Khongphatthanayothin, M.D.

Division of Cardiology, Department of Pediatrics

King Chulalongkorn Memorial Hospital

1873 Rama IV Rd., Patumwan, Bangkok 10330, THAILAND

Tel: (+66-9) 206-0384; FAX : (+66-2) 256-4911, (+66-2) 714-8524

Email: apichaik@yahoo.com

Running Title: Portal blood flow and liver enzyme elevation in dengue virus infection

Key words: Dengue, Dengue hemorrhagic fever, Dengue shock syndrome, Portal blood flow, Liver injury

Abstract

To find out if liver injury in patients with dengue virus infection is caused by ischemia (ischemic hepatitis), we studied portal blood flow in 36 serologically-confirmed dengue virus infection during the toxic stage. The patients' age was 10.9±2.5 years and 13 had dengue fever, 10 dengue hemorrhagic fever and 13 dengue shock syndrome. Plotting of ultrasound-derived portal blood flow against the level of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) demonstrate a "threshold effect" of portal venous blood flow to the elevation of liver enzyme. All patients with significantly elevated AST and ALT (> 200 IU/mL) had portal venous flow of less than 300 mL/min/m^2. Some patients, however, was protected from liver injury despite low portal venous blood flow. We concluded that liver injury in patients with dengue virus infection could be partly explained by ischemia, with the possibility of other factors contributing to the different degree of liver injury. Alteration of the splanchnic circulation may be an important pathophysiologic process in patients with dengue virus infection.

Background

Liver involvement in patients with dengue virus infection is well known. Liver enzyme elevation was noted in 45-98% of patients with dengue virus infection[1-5] with more elevation in patients with higher severity of the illness[6,7]. Liver failure due to dengue virus infection is well documented in the literature[8-11] and dengue virus is the most common cause of liver failure in Thai children in the current era[12].

The pathogenesis of liver involvement in DHF and DF is not well understood. Potential explanation includes direct infection of hepatocyte[13], hypoperfusion of liver from shock, and sinusoidal endothelial damage[14]. Because liver enzyme elevation tended to be more severe in patients with higher severity of the illness (i.e. in shock cases more than non-shock cases), we hypothesized that hepatic hypoperfusion may be the etiology for liver injury in this disease. The objective of this study was to correlate the degree of hepatic hypoperfusion with that of liver injury. Because portal vein supplies the majority of hepatic blood flow and its ease of measurement compared to hepatic arterial flow, we use portal blood flow as the surrogate for hepatic perfusion. Liver enzymes (alanine aminotransferase, ALT and aspartate aminotransferase, AST) was used to quantify the degree of livery injury.

Methods

1: Subjects

Convenient samples of children (less than 14 years) who were admitted with presumptive diagnosis of dengue virus infection at King Chulalongkorn Memorial Hospital were enrolled. Only patients with serologic confirmation of dengue virus infection were included in the final analysis.

2. Procedures

After written inform consent was obtained, blood samples were collected for serologic and/or PCR confirmation of dengue virus infection. On the day of defervescence, blood sample for determination of Aspartate aminotransferase (AST) and alanine amiotransferase (ALT), and ultrasonographic studies of portal vein were done. On the day of discharge, ultrasonographic study of the right pleural cavity was performed for evidence of capillary leakage (pleural effusion). Another blood specimen was collect for paired determination of dengue serology.

3. Ultrasonographic study of portal blood flow:

Ultrasonographic studies of portal vein was done by one investigator (A.K.) using Aloka Prosound SSD5500 (Aloka Inc., Tokyo, Japan) imaging system using 3-5 MHz transducer with electrocardiogram attached. The size of portal vein was measured in supine right subcostal position, with the vein seen longitudinally. The maximal diameter of portal vein was obtained just distal to the site where the hepatic artery crossed the vein. All measurements were done from inner wall to inner wall, during expiration, and at before QRS complex on the electrocardiogram. An average from three measurements was used for further analysis.

The velocity of blood flow in the portal vein was measured by Doppler interrogation of the right portal vein just before its first intrahepatic branching. Transducer was placed at right lateral aspect of the upper abdomen in supine position such that the right portal vein was seen pointing toward the transducer during expiration. The mean velocity of portal vein flow was obtained during respiratory hold in expiration, and averaged for 3 cardiac cycles. In patients who cannot hold respiration reliably (in small children), averaged value from 3 respiratory cycles was used. Doppler measurements were done 3 times and the value averaged. Only Doppler interrogation with less than 45 degrees angle between the ultrasound beam and the vessel was accepted. Angle correction was used for all Doppler measurements. Mean Doppler velocity of blood flow was calculated by the software provided with the ultrasound machine with automatic tracing of the Doppler spectral signal.

Portal blood flow was calculated as the cross-sectional area of the portal vein (π x [dameter/2]^2) multiply by the velocity of the right portal vein.

4. Confirmation of dengue virus infection

Serologic study of dengue virus infection was done at The Arm Force Research Institute of Medical Sciences (AFRIMS, Bangkok, Thailand) using ELISA method. The laboratory procedures and interpretations were previously reported[15]. Positive PCR test for dengue virus was accepted as an evidence of dengue virus infection in patients who did not have ELISA results due to missing specimen or lack of paired specimens. Nonserotype-specific reverse transcription - nested polymerase chain reaction (RT-nested PCR) was performed at the Division of Infectious Disease, Department of Medicine, Chulalongkorn University School of Medicine using consensus primers targeting 3'-untranslated region of all dengue viruses. The sensitivity and specificity of the test has been determined to be 86.8 and 100% using plasma or serum specimens, respectively [16].

5. Clinical data

Diagnosis and grading of dengue hemorrhagic fever (DHF) was done according to the criteria published by the World Health Organization (WHO)[17]. Diagnosis of DHF required at least 1 evidence of capillary leakage (rising of hematocrit > 20% or presence of pleural effusion and/or ascites). Dengue shock syndrome (DSS) was diagnosed when the patient with DHF had clinical findings of shock as: 1) hypotension for age and cold clammy skin or restlessness or 2) narrow pulse pressure ($\leq$ 20 mmHg.) and rapid and weak pulse[17]. Ultrasonographic study of the right pleural cavity was used to detect the presence of pleural effusion during the convalescent stage in all cases. Treatments including fluid administration of all patients were given by house staff and attending staff of our department according to the guideline published by WHO[17]. (http://www.who.int/csr/resources/publications/dengue/Denguepublication/en/)

6. Ethic committee approval and patient's consent

The study was approved by the Ethic Committee of the Faculty of Medicine, Chulalongkorn University. Written informed consent was obtained from each subject and/or appropriate guardian prior to enrollment.

Results

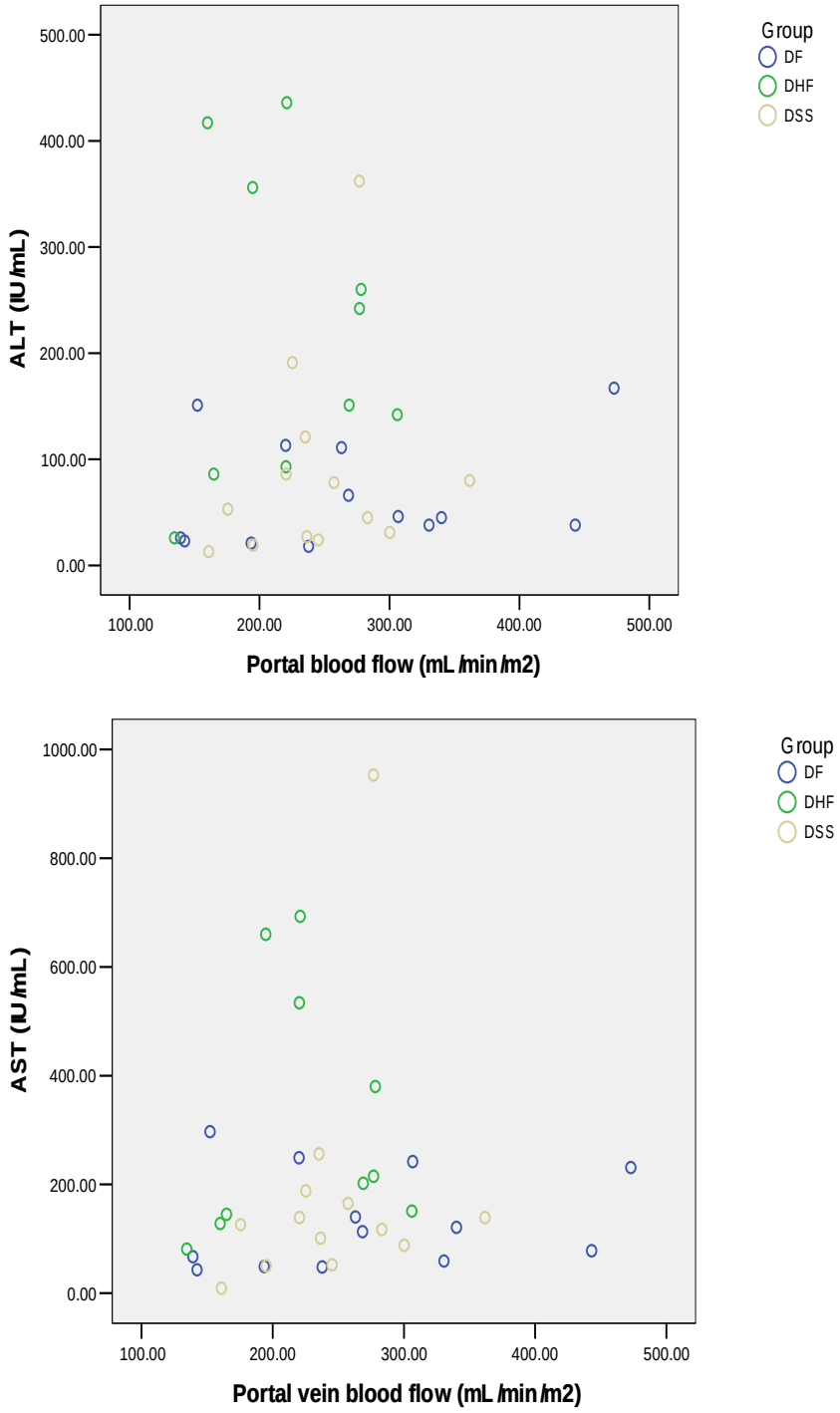
Thirty-six patients (20 males, 16 females, mean age 10.9±2.5 years) were enrolled. There were 13 patients with dengue fever, 10 with dengue hemorrhagic fever without shock and 13 with dengue shock syndrome. The clinical data of all patients based on severity are summarized in Table 1.

Table 1: Demographic and clinical data of all patients based on the severity of dengue virus infection

	Dengue fever (DF)	Dengue hemorrhagic fever without shock (DHF)	Dengue shock syndrome (DSS)
Age (years)	9.9 ± 2.4	12.0 ± 2.3	11.1 ± 2.6
Sex (male/female)	6/7	5/5	9/4
Maximum hematocrit (%)	40.2 ± 3.4	45.0 ± 2.6	46.7 ± 6.4
Minimum Platelet (x 10^3 per mm^3)	75.5 ± 22.5	33.0 ± 17.3	28.5 ± 17.3

The relationship between portal vein blood flow (PBF) and liver enzyme elevation in patients with dengue virus infection is demonstrated in figure 1.

Figure 1: Scatterplot demonstrating the relationship between portal blood flow (X axis) and liver enzyme (ALT, top; and AST, bottom) in patients with dengue virus infection.



Discussion

Liver injury has long been recognized in patients with dengue virus infection[1-12]. While it is well documented that the incidence of liver enzyme elevation in patients with dengue virus infection is dependent upon the severity of the illness (higher with more severe diseases), its etiology is still a matter of speculation[6,7]. Histopathologic studies of liver in these patients often demonstrated hepatocyte swelling with intracellular inclusion bodies and focal areas of paracentral necrosis, hepatic congestion, and sinusoidal congestion[18-20]. Kupffer cell swelling and occasional dislodge of cells into sinusoidal space has been described[18]. Contrary to most other viral hepatitis, evidence of white blood cell infiltration of the liver has not been impressive. While these findings were mostly non-specific, hepatic injury in patients with dengue virus infection is believed not likely to be the same as others viral-induced hepatitis.

Central into the debate of the etiology of liver injury in patients with dengue virus infection is whether liver injury is caused by hepatic hypoperfusion. To answer this question, we initially examined the relationship between hemodynamic parameters measured by echocardiogram and the degree of liver enzyme elevation in these patients. The results showed a scatter relationship between cardiac index and the level of ALT and AST. Because of our previous study showing that various degree of liver congestion occurred in patients with dengue virus infection[21], we hypothesized that hepatic blood flow may not always correlate with total body blood flow in these patients because of possible downstream impediment of the hepatic blood flow caused by liver congestion. We therefore examined the relationship between hepatic blood flow and the degree of liver enzyme elevation as shown in this study. Because of its ease of measurement by ultrasound and its role as the major source of hepatic blood supply, portal venous blood flow was used as the surrogate for hepatic blood flow. Because ALT and AST are highest at the toxic stage (Figure 1), we use the level drawn on the day of defervescence as the surrogate for liver injury.

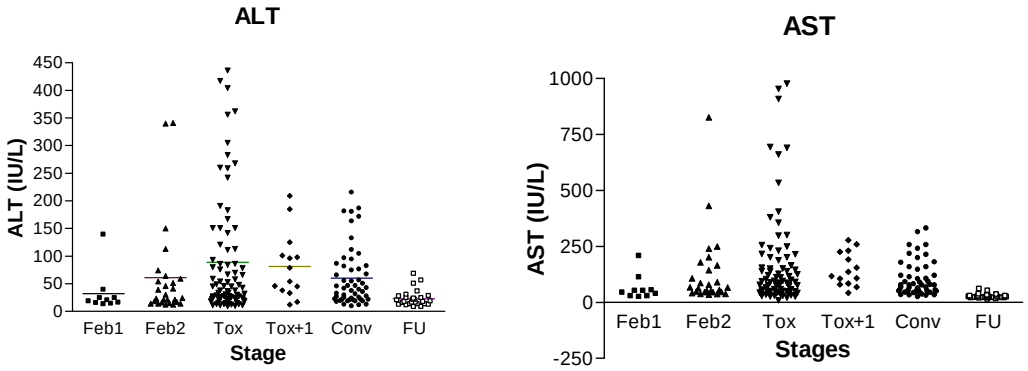


Figure 2: Levels of ALT and AST during different stages of dengue virus infection. Feb1 = Febrile stage, early days, Feb2 = the day just before defervescence, Toxic = at defervescence, Conv = the day before discharge, FU = at follow-up. Khongphatthanayothin, et al, unpublished

From Figure 1, it was apparent that all patients with significant elevation of ALT or AST (> 200 IU/mL) had portal blood flow of less than 300 mL/min/m^2. However, not all patients with "low" portal blood flow had elevation of AST and/or ALT. This can be explained by dual sources of blood supply common to hepatic physiology. Hepatic arterial supply may prevent liver from ischemic insult in many patients with low portal blood flow. There could also be other factors contributing to various degree of hepatic tolerance to ischemic insults among different patients, such as the degree of hepatic congestion, the length of hypotension and adequacy of fluid resuscitation, etc. Different degree of direct hepatic injury caused by dengue virus infection, in combination with hemodynamic factors is another explanation for lack of liver injury in some patients with low portal blood flow.

From the literature, liver injury caused by hypotension and shock has been described as "ischemic hepatitis" or "shock liver"[22-24]. Although hypotension and/or low cardiac output are required for the diagnosis of ischemic hepatitis, many patients with shock and/or cardiac arrest do not have this condition[22]. One study identifies passive hepatic congestion as the main risk factor for liver injury associated with shock[22]. Because liver congestion is common in patients with dengue virus infection, we believe ischemic hepatitis could be the reason for liver enzyme elevation in some patients. Other similarities of dengue-associated liver injury and ischemic hepatitis included marked rise in the level of AST[25], rapid rise and fall of ALT and AST[24-27] and liver pathology of centrilobular necrosis[27].

In conclusion, we demonstrated that all dengue virus infected patients with significantly elevated liver enzyme had low portal blood flow. Liver injury in some patients with dengue virus infection could therefore be explained by decreased hepatic perfusion coupled with liver congestion and/or other unknown contributing factors. The changes in splanchnic and hepatic circulation in patients with dengue virus infection may be an important pathophysiologic process for dengue-related illness.

Acknowledgement:

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Manuscript 7

Correlation between echocardiographic derived hemodynamic variables and capillary lactate during toxic stage of dengue hemorrhagic fever

Sarawut Pinta, Soravit Pongpittayut, Kanyalak Sethkrikul, Vithawas La-ookhun, Pornthep Lertsapcharoen, Apichai Khongphatthanayothin.

Department of Pediatrics, Faculty of Medicine, Chulalongkorn University and King Chulalongkorn Memorial Hospital, Bangkok, Thailand

This study was supported by The Thailand Research Fund (Grant# RMU 4980019).

Address for correspondence:

Apichai Khongphatthanayothin, M.D.

Division of Cardiology, Department of Pediatrics

King Chulalongkorn Memorial Hospital

1873 Rama IV Rd., Patumwan, Bangkok 10330, THAILAND

Tel: (+66-9) 206-0384; FAX : (+66-2) 256-4911, (+66-2) 714-8524

Email: apichaik@yahoo.com

Key words: Dengue, Dengue hemorrhagic fever, Dengue shock syndrome, Echocardiography, Hemodynamic monitoring

Abstract

To find the usefulness of a single echocardiographic determination of the hemodynamic status at the toxic stage for predicting the adequacy of the circulation in patients with dengue virus infection, 20 patients (12 boys and 8 girls, mean age 11.0 ± 2.9 years) were enrolled in this study. Echocardiographic determinations of cardiac index and end-diastolic volume index were performed within 6 hours of defervescence and data were correlated with 1) capillary lactate at the time of echocardiogram, 2) capillary lactate 12 hours later, and 3) the change of capillary lactate within the 12 hours. Twelve patients had dengue fever, 5 had dengue hemorrhagic fever without shock and 3 had dengue shock syndrome. No correlation was found between the cardiac index or end-diastolic volume index and lactate/change of lactate in this group of patient. It was concluded that single determinations of cardiac index or end-diastolic volume index in patients with dengue virus infection at toxic stage were not predictive of the lactate at that time nor the change thereafter.

Background

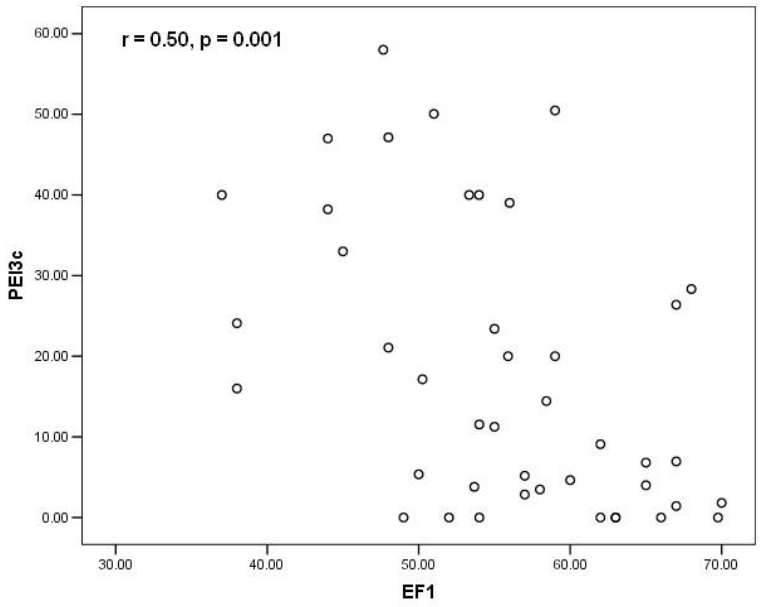
Dengue virus infection is one of the most important emerging infectious diseases affecting children in tropical countries[1, 2]. The disease is endemic in all provinces of Thailand and in many tropical parts of the world, such as in Southeast Asia, India, Central and South America, and parts of Africa and Australia[2]. It is now considered to be a global pandemic, with recorded prevalence in 101 countries around the world. The estimate for an annual global infection is currently 50-100 million patients, with an annual incidence of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), the two most serious forms of this disease, of 250000-500,000 patients each year[2]. Dengue virus infection is considered to be a major public health problem in Thailand[3].

While the actual pathophysiologic process of DHF remains unclear, certain clinical presentations have been well described[3, 4]. In susceptible patients, dengue hemorrhagic fever develops 2-9 days after symptomatic infection by dengue virus. Shock and bleeding are the main serious clinical presentations of DHF, both of which can lead to a serious morbidity or death. The pathophysiologic process is self-limited and surviving patients recover spontaneously in 24-48 hours. The mainstay of treatment for DHF remains supportive, with meticulous care for shock and bleeding during the toxic stage of the disease.

The mainstay of treatment for low cardiac output state and shock in patients with DHF/DSS is intravenous fluid[3, 4]. Meticulous care is needed when giving intravenous fluid to patients with DHF/DSS since over- treated patients may develop fluid overload as a complication[3, 4]. Currently, clinical examination and hematocrit are used to monitor the treatment in patients with DHF. However, clinical examination can be unreliable and difficult in certain patients such as in obese children. Hematocrit monitoring can be misleading in patients with significant bleeding and may not be entirely reliable because of its insensitivity to venous pooling. Echocardiogram is ideal as a monitoring tool in patients with severe shock or in whom the clinical examination is difficult because of its non-invasiveness, portability and the ability to assess intravascular volume and cardiac contractility at the same time. Assessment of cardiac contraction may be important in cases with severe shock since our data demonstrated the association between cardiac contraction (ejection fraction) and the degree of pleural effusion after treatment (figure 1). Echocardiogram has been proved to be at least as good as invasive monitoring in patients with septic shock[5, 6]. The equipment is now widely available in most provincial hospitals in Thailand and successful limited training of non-cardiologist physicians for the purpose of hemodynamic monitoring has been demonstrated to be possible in emergency[7, 8] and intensive care settings[9, 10]. To our

knowledge, the utility of echocardiogram as hemodynamic monitoring for low cardiac output state and shock in patients with DHF/DSS has never been previously studied.

Figure 1: Scatterplot showing the relationship between left ventricular ejection fraction (EF1) and the amount of pleural effusion (PEI3c, % of hemithorax) after treatment of DHF.



The purpose of this study was to evaluate the correlation between echocardiographic derived hemodynamic variables and the degree of hypoperfusion assessed by capillary lactate in patients with dengue virus infection during the toxic stage.

Methods:

Subjects: Children (age 0-15 years) with ELISA or PCR-proven DV infection who were admitted to King Chulalongkorn Memorial Hospital (Bangkok, Thailand) were enrolled. We excluded patients with underlying heart or metabolic diseases which may affect blood lactate level.

Lactate determination: Capillary blood was used for lactate determination by a hand-held device (Accutrend Lactate, Roach Diagnostics, Germany) within 6 hours after defervescence (toxic stage) and 12 hours thereafter. Lactate level of $\leq$ 2.2 mmol/L was considered to be normal[11].

Echocardiography

Echocardiographic studies were performed by our investigators (SP,KS) using Aloka Prosound SSD5500 echocardiographic machine (Aloka Inc., Tokyo, Japan) at febrile, toxic, convalescent stages and at follow up. An M-mode scan of left ventricle from the standard parasternal long-axis view, at the level of mitral valve tip was recorded on tape simultaneously with the EKG and phonocardiogram. The recordings were later reviewed and measured by one investigator (SP) who was blinded to the clinical data of the patients, using the software provided with the echocardiographic machine. Three consecutive cardiac cycles were analyzed for each variable and the averaged values were used for further analysis.

Measurements of left ventricular walls and dimensions were done according to the previously published guideline[12]. End-diastole was defined as the time at the onset of QRS complex and end-systole was defined as the time at the first high frequency component of the second heart sound (S2) on phonocardiogram. Left ventricular volumes at end-diastole (EDV) and end-systole (ESV) were calculated by the method of Teichholz's[13]. Ejection fraction was calculated as 100 x (EDV-ESV)/EDV.

Determination of pleural effusion:

The amount of right pleural effusion during the convalescent stage was determined by ultrasonographic examination of the right pleural space as previously described[14]. Pleural effusion index (PEI) was calculated as the width of right pleural effusion divided by the width of the patient's hemithorax at level of the xiphoid process.

Clinical data:

The progression of the patient's illness was classified into three clinical stages as febrile, toxic and convalescent stages and as dengue fever (DF), dengue hemorrhagic fever without shock (DHF) and dengue shock syndrome (DSS) according to the criteria published by the World Health Organization (WHO)[4]. All patients were treated by our house staff and/or attending staff according to the WHO guideline. Lactate levels were not known to the physician who treated the patient.

Variables and Statistics:

Data are expressed as mean ± SD. Unpaired t-test or one-way ANOVA were used to compare continuous variables and Chi-square test was used to compare categorical variables

among 2 or more groups. Correlation between 2 continuous variables was assessed by linear regression.

Ethic committee approval and patient's consent

The study was approved by the Ethic Committee of the Faculty of Medicine, Chulalongkorn University and Sawanpracharak Hospital. Written informed consent was obtained from each subject and/or appropriate guardian prior to enrollment.

Results

Twenty patients (12 boys and 8 girls, mean age 11.0 ± 2.9 years) were enrolled. The demographic and clinical data of all patients are summarized in Table 1.

Variables	**Groups**			***p***
	DF (n = 12)	**DHF,no shock (n = 5)**	**DSS (n = 3)**	
Age	11.4 ± 3.1	10.2 ± 2.8	11.0 ± 2.8	.772
Maximal Hct	41.2 ± 2.7	46.5 ± 3.5	46.7 ± 4.0	.006
Minimal Plt	61.8 ± 22.9	25.4 ± 5.3	60.7 ± 40.9	.026
Lactate (mmol/L) (defervescence)	2.2 ± 0.6	2.4 ± 0.5	3.0 ± 0.6	.156
Lactate (mmol/L) (12 hours later)	2.2 ± 0.8	2.7 ± 1.0	2.6 ± 0.5	.551
IV fluid given in 12 hours (mL/m2)	415 ± 142	839 ± 541	1332 ± 383	.001
PEI (%)	0	8.1 ± 8.0	10.1 ± 11.5	.010

The relationship between cardiac index at toxic stage in these 20 patients and 1) lactate at deferveacence, 2) lactate 12 hours after IV fluid resuscitation, and 3) the change of lactate are showed in Figure 1. There appeared to be no relationship between cardiac index and lactate data, both with the absolute data and with the change of lactate during the 12 hours after defervescence.

The relationship between and end-diastolic volume at toxic stage in these 20 patients and 1) lactate at defervescence, 2) lactate 12 hours after IV fluid resuscitation, and 3) the change of

lactate are showed in Figure 2. There appeared to be no relationship between end-diastolic volume index and lactate data, both with the absolute data and with the change of lactate during the 12 hours after defervescence.

Discussion

Contrary to our original hypothesis, we could not demonstrate any relationship between hemodynamic data taken from a single echocardiogram at toxic stage and the level of lactate or subsequent changes. These results were unexpected. Some of our explanations are as follow:

1. Echocardiogram was done only 1 time. As hemodynamic status in these patients is dynamic and could change significantly during the 12 hours of IV fluid resuscitation due to ongoing fluid shift across from intravascular compartment to extravascular compartment or due to the variation of IV fluid resuscitation which were not strictly controlled. As such, the early determination of hemodynamic data might not keep up with the subsequent changes and thus might not be correlate with lactate or the rate of change in lactate.
2. Echocardiographic measurements and calculations of end-diastolic volume or cardiac index might have been subjected to a wide variation, both between echocardiographer and between reader of the recorded sonogram.

In conclusion, we did not find any correlation between echocardiographic determination of cardiac index or left ventricular end-diastolic volume and the degree of tissue hypoperfusion assessed by capillary lactate or its subsequent changes. The use of single echocardiographic assessment of hemodynamic status in patient with dengue virus infection at toxic stage may not be useful in predicting the current level of lactate or the subsequent changes. Further study on multiple assessment by echocardiogram might yield a different results and could be the subject for further investigation.

Acknowledgement

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Figure 1: The relationship between cardiac index at toxic stage and 1) lactate at defervescence, 2) lactate at 12 hours after IV fluid resuscitation, and 3) the change of lactate within the next 12 hours.

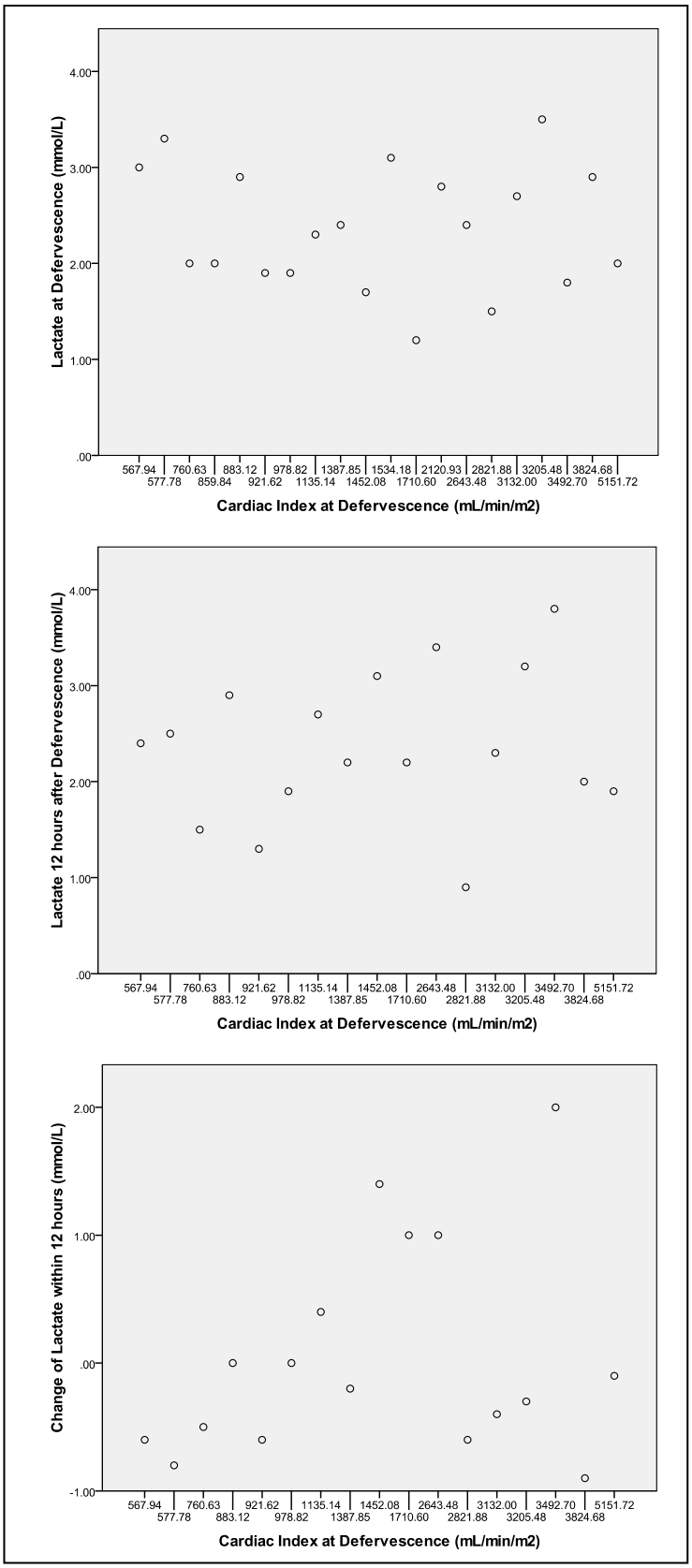
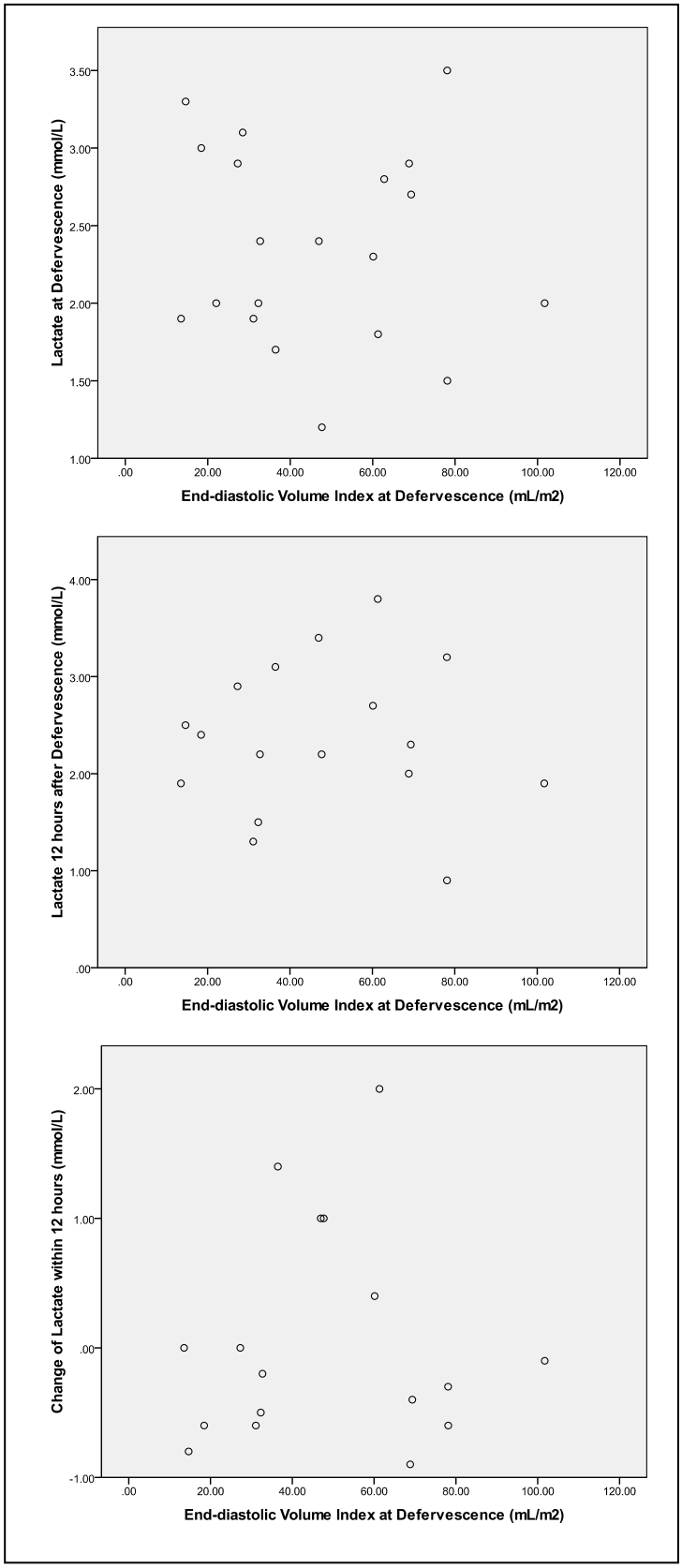


Figure 2: The relationship between end-diastolic volume index and 1) lactate at defervescence, 2) lactate at 12 hours after IV fluid resuscitation, and 3) the change of lactate within the next 12 hours.



Reversed Direction of Portal Venous Blood Flow in a Patient with Dengue Virus Infection and Fatal Liver Failure

Apichai Khongphatthanayothin, M.D. [1], Atchara Mahayosnond, M.D. [2], Yong Poovorawan, M.D.[1]

1 = Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

2 = Department of Radiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

Keywords: Dengue, Dengue Hemorrhagic Fever, Liver Failure, Ultrasonography, Children

Address for correspondence:

Apichai Khongphatthanayothin, M.D.

Department of Pediatrics

King Chulalongkorn Memorial Hospital

1873 Rama IV Rd. Patumwan, Bangkok 10330, THAILAND

Tel: (+6689) 155-5545; FAX :(+662) 256-4911,

Email: apichaik@yahoo.com

List of Abbreviations

BP = Blood Pressure, BUN = blood urea nitrogen, Cr = serum creatinine, Hb = hemoglobin, Hct = hematocrit

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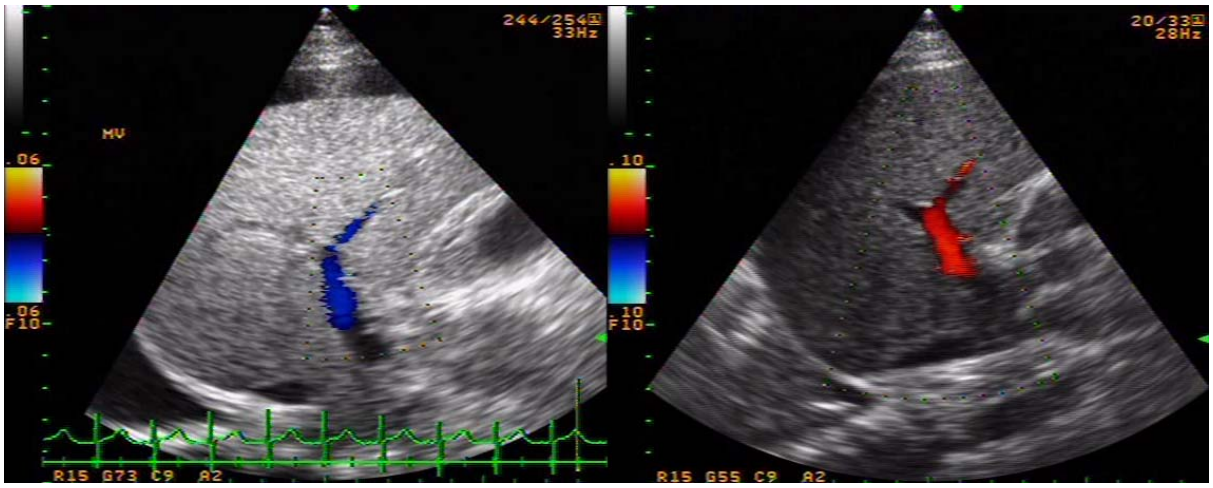
A 10-year old boy presented with 4 days history of high fever, malaise, headache, and vomiting. At admission, he was in shock (BP=80/40 mmHg). Enlarged liver (4 cm) and generalized petechiae were found on physical exam. Dengue shock syndrome was diagnosed based on positive dengue IgM and negative blood culture for bacteria. Laboratory examination showed WBC=14,930 cells/mm^3, Hb=16.4 g/dL, Hct=48.2%, Platelet=18,000/mm3, BUN=33 mg/dL, Cr=1 mg/dL, Na^+=128 meq/L, K^+=6.2 meq/L, Cl^-=91 meq/L, total CO_2=5 meq/L, total billirubin=6.9 mg/dL, direct billirubin=3.9 mg/dL, SGOT=3,507 U/L, SGPT=2,775 U/L, PT=43 sec (INR=3.4) and PTT=93.5 sec (control 28.7 sec). The hospital course was complicated by liver failure, renal failure, gastrointestinal hemorrhage and hypoglycemia. Although hemodynamic condition stabilized over 24 hours, the patient continued to have further rise of SGOT (11,690 U/L), SGPT (4,490 U/L), total billirubin (34.8 mg/dL), and direct billirubin (18.4 mg/dL) during the next 6 days. Hepatic ultrasound on the second day after admission showed totally reversed direction of portal venous blood flow away from the liver (Figure 1, left) which was changed to bi-directional direction on the next day and finally to normal direction (although with low velocity) 3 days later (Figure 1, right). There was no obstruction of hepatic veins. The patient expired 6 days after admission due to pulmonary hemorrhage and progressive respiratory failure.

Dengue virus infection is one of the common causes of acute liver failure in children in Thailand and tropical countries(1). The cause of liver failure in this disease is still unknown. The ultrasonographic finding of reversed portal blood flow in our patient

with fatal liver failure was different from increased portal blood flow seen in patients with acute liver failure from paracetamol poisoining or viral hepatitis(2). Ultrasonographic findings of ascites, hepatomegaly, swollen gall bladder and decreased portal venous flow velocity in this disease resemble that of hepatic venoocclusive disease which, in fatal case, can also present with hepatofugal portal flow(3). We postulated that sinusoidal obstruction of portal blood flow may be an important pathophysiologic process in this disease that should be further studied.

Figure 1: Left=ultrasonography with color flow Doppler of the liver on the second day of admission showing reversed direction of blood flow in the right branch of portal vein (hepatofugal). There was diffuse increased liver parenchymal echo, swelling of the gallbladder wall and right pleural effusion. Right=returning of the normal direction of portal venous flow (hepatopetal) 3 days later. Liver parenchymal echo changed to normal. Pleural fluid and swelling of the gallbladder wall also disappeared.



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Output

A. Accepted for publication

1. Sathupan P, Khongphattanayothin A, Srisai J, Srikaew K, Poovorawan, Y. The role of vascular endothelial growth factor (VEGF) leading to vascular leakage in children with dengue virus infection. ***Ann Trop Paediatr 2007;* 27: 179-184.**
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B. Submitted, under review

1. Rungteeranon N, Supachokechaiwattana P, Vithessonthi K, La-orkhun K, Lertsapcharoen P, Khongphatthanayothin A. Capillary Lactate as a Potential Hemodynamic Monitoring Tool in Patients with Dengue Virus Infection. Abstract accepted for Mini-oral presentation at The 6th World Congress of Pediatric Critical Care Medicine, Sydney, Australia, March 2011. Manuscript submitted for publication in ***Indian J Crit Care Med***
2. Khongphatthanayothin A, Mahayosnond A, Poovorawan P. Reversed Direction of Portal Venous Blood Flow in a Patient with Dengue Virus Infection and Fatal Liver Failure. Submitted for ***Hepatology***

3. Khongphatthanayothin A, Ratanasukol P, Supachokechaiwattana P, Sethkrikul K, Poovorawan Y. Relationship between portal blood flow and liver enzyme elevation in patients with dengue virus infection: Is liver injury a result of hepatic hypoperfusion? Submitted for ***Clinical Infect Dis*** (Abstract presented as Oral Presentation at The 6th World Congress of Pediatric Critical Care Medicine, Sydney, Australia, March 2011)

ภาคผนวก

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The role of vascular endothelial growth factor leading to vascular leakage in children with dengue virus infection

PATCHARAPA SATHUPAN*†, APICHAI KHONGPHATTANAYOTHIN†, JIRABAT SRISAI†‡, KARAKADE SRIKAEW†‡ & YONG POOVORAWAN†

*Departments of Paediatrics, *Thammasat University, Patumthani, †Chulalongkorn University, Bangkok, and ‡Had Yai Hospital, Songkhla, Thailand*

(Accepted May 2007)

Abstract Increased vascular permeability is the main aetiology for hypovolaemic shock and circulatory failure in dengue haemorrhagic fever (DHF).
Aim: To investigate the role of vascular endothelial growth factor (VEGF) in the pathogenesis of DHF.
Methods: Serum samples from 41 patients [15 dengue fever (DF), 26 DHF] with serologically confirmed dengue virus infection during the febrile, toxic, convalescent stages and at follow-up were analysed for VEGF. Plasma samples from an additional 27 children (16 DF and 11 DHF) during the febrile, toxic stages and at 4-week follow-up and from eight healthy controls were analysed for VEGF.
Results: Serum and plasma VEGF levels were not elevated during the febrile or toxic stages of dengue virus infection and did not differ between patients with DF and DHF.
Conclusion: Plasma leakage in patients with DHF cannot be explained by elevation of VEGF during the toxic stage of the illness.

Introduction

Dengue haemorrhagic fever (DHF) is one of the most important infectious diseases in Thailand and many other tropical regions of the world. Most patients infected by the dengue virus are asymptomatic. Presentations in those with symptoms are classified into three groups: undifferentiated fever, dengue fever (DF) and DHF. Dengue shock syndrome (DSS) is a severe complication of DHF, characterised by a massive increase in vascular permeability leading to hypovolaemic shock and circulatory failure. Evidence of vascular leakage are haemoconcentration, ascites, pleural effusion, hypo-albuminaemia and low central venous pressure. The pathogenesis of vascular leakage in DHF is not clearly understood. Release of cytokines and chemokines from monocytes and macrophages which target endothelium are believed to play a key role in the pathogenesis of the vascular leakage.[1,2]

Vascular endothelial growth factor (VEGF), originally named vascular permeability factor, is a homodimeric heparin-binding glycoprotein with potent angiogenic, endothelial-cell-specific mitogenic and vascular permeability-enhancing activities. Several studies have shown that VEGF plays a role in the capillary hyperpermeability that characterises ovarian hyperstimulation syndrome,[3–5] pre-eclampsia,[6] cirrhosis with spontaneous bacterial peritonitis (SBP)[7] and post-operative capillary leak syndrome in children undergoing cardiopulmonary

Reprint requests to: Dr Apichai Khongphatthanayothin, Department of Paediatrics, King Chulalongkorn Memorial Hospital, 1873 Rama IV Road, Patumwan, Bangkok 10330, Thailand. Fax: +66 2 256 4911; e-mail: apichaik@yahoo.com

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bypass.[8] These findings prompted us to determine the possible role of VEGF in the pathogenesis of DHF/DSS.[9,10]

Subjects and Methods

Subjects

Between 2003 and 2005, 41 patients aged 5–15 years admitted to King Chulalongkorn Memorial Hospital with suspected dengue viral infection during the febrile stage were enrolled. After receiving informed consent, blood samples were collected for determination of serum VEGF levels at four stages: febrile (1st day of enrolment), toxic (day of defervescence), convalescent (discharged date) and recovery (at follow-up, approximately 4–6 weeks after the onset of fever). The patients' illnesses were classified as DF or DHF according to World Health Organization criteria.[1] Patients with no serological confirmation of dengue virus infection were excluded.

To exclude the possible effect of platelets on serum VEGF levels, plasma samples were collected from another 27 patients admitted to Had Yai Hospital with serologically confirmed dengue viral infection during 2005–2006 for determination of the plasma VEGF levels during the febrile and toxic stages and at follow-up. In addition, plasma samples were obtained from eight healthy control children (aged 8–14 years) for determination of VEGF.

The studies were approved by the ethics committee of the Faculty of Medicine of Chulalongkorn University. Written informed consent was obtained from each subject and/or appropriate guardian before enrolment.

Serological confirmation of dengue viral infection was undertaken at The Armed Force Research Institute of Medical Sciences (AFRIMS, Bangkok) by enzyme-linked immunosorbent assay (ELISA).

Measurement of VEGF

Three millilitres of blood (using EDTA as anticoagulant for plasma samples) were collected and centrifuged at approximately 1000 G. The plasma/serum was then removed and stored at $\leqslant -70^{\circ}$C for subsequent analysis.

Plasma and serum VEGF levels were measured by solid-phase ELISA (Quantikine R&D Systems, Minneapolis, MN, USA), designed to measure VEGF165 levels in cell culture supernatant, serum and plasma.

Data collection and analysis

The data were analysed using SPSS for Windows (version 14) software. Continuous variables with normal distribution were expressed as means (SD) and compared using one-way analysis of variance (ANOVA). Categorical variables were compared using the χ^2 test or Fisher's exact test, as appropriate. Because VEGF levels and platelet counts were not normally distributed, comparison of these variables in DF and DHF and during the course of the disease was undertaken using non-parametric methods (Kruskal–Wallis or Mann–Whitney U tests). A p-value of <0.05 was considered significant.

Results

Demographic data

Forty-one patients (16 females and 25 males) were enrolled for serum VEGF determination. The mean (SD) age was 11.1 (3.1) years. There were 15 children with DF and 26 with DHF (14 DHF without shock and 12 DSS).

Of the 27 patients enrolled for plasma VEGF determination, eight were female and 19 male, and mean (SD) age was 9.8 (2.5) years. Sixteen children had DF and 11 DHF (ten without shock and one with shock). Eight healthy children [mean (SD) age 8.75 (1.63) years] served as controls.

In neither the serum nor the plasma group was there a significant difference in age, gender or day of fever between patients with DF and those with DHF (Table 1).

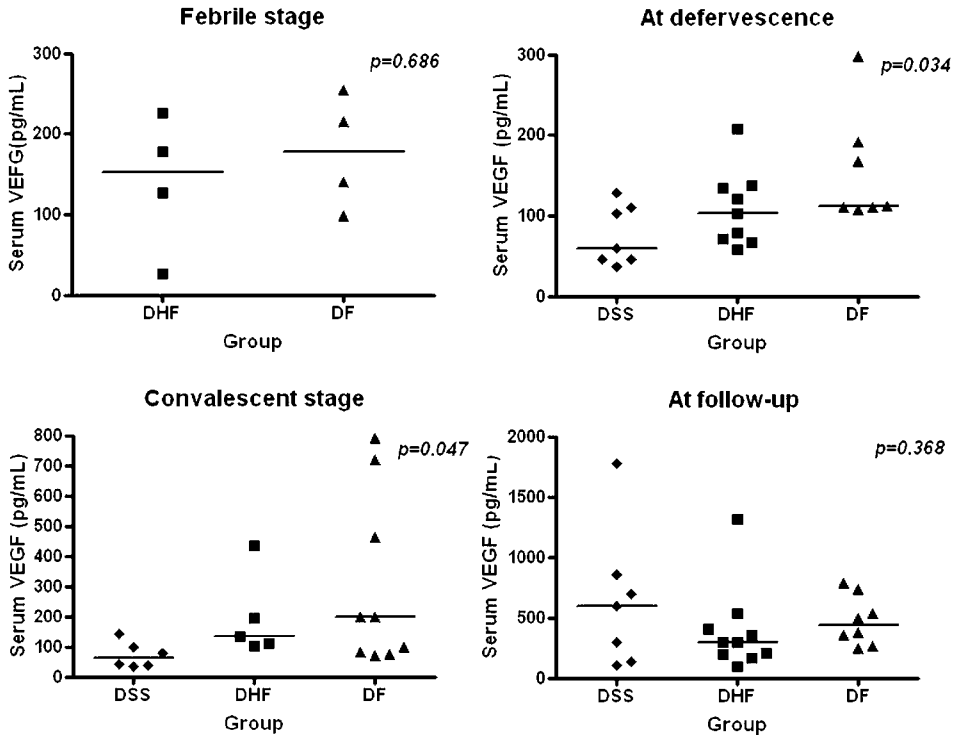

FIG. 1. The difference in serum VEGF levels between groups (DF, DHF, DSS) during each stage of dengue virus infection. The horizontal lines indicate the median VEGF value for each group.

Difference in serum and plasma VEGF levels between patients with DF vs DHF

Serum levels of VEGF in patients with DF, DHF and DSS during different stages of dengue virus infection are shown in Fig. 1. A trend towards lower VEGF levels in patients with more severe dengue virus infection was observed during the febrile, toxic and convalescent stages, but not on follow-up.

Plasma VEGF levels in patients with DF and DHF and in the controls during the febrile, toxic and recovery stages of the disease are shown in Fig. 2. Similar to serum VEGF, plasma VEGF levels were

TABLE 1. *Demographic and clinical data.*

	Serum group			Plasma group		
	DF, $n=15$	DHF, $n=26$	p-value	DF, $n=16$	DHF, $n=11$	p-value
Male:female	8:7	17:9	0.517	9:7	10:1	0.090
Age (SD), yrs	10.5 (3.3)	11.5 (3.1)	0.393	9.5 (2.3)	10.2 (2.8)	0.495
Duration of fever, days	5.1 (1.2)	5.0 (0.8)	0.928	4.0 (1.1)	3.8 (1.1)	0.673
Maximum Hct (%)	40.2 (3.9)	45.2 (4.9)	0.001	41.0 (3.0)	46.0 (4.1)	0.001
Lowest platelet count ($\times 10^9$/L)*	94.5 (72.5–101.5)	35.0 (29.0–60.0)	0.004	82.5 (59.5–102.8)	59.0 (32.0–73.0)	0.110

DF, dengue fever; DHF, dengue haemorrhagic f ever; Hct, haematocrit; IQR, interquartile range. * Median (IQR).

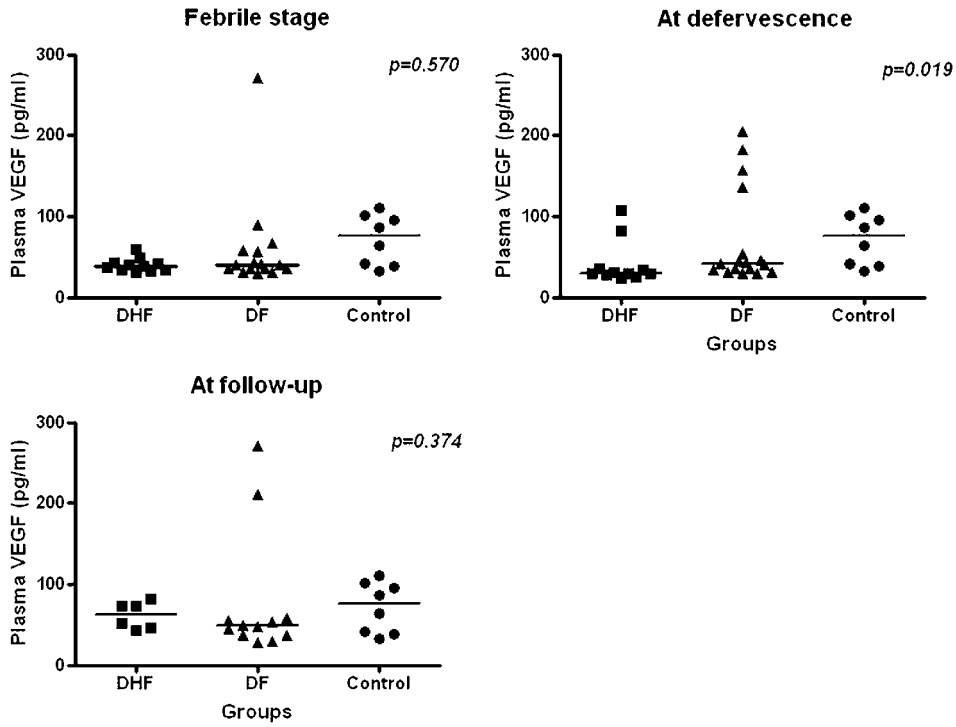

FIG. 2. The difference in plasma VEGF levels between groups (DF, DHF, controls) during each stage of dengue virus infection. The horizontal lines indicate the median VEGF values.

lower in patients with DHF than with DF at the febrile and toxic stages. The difference between plasma VEGF in patients with DHF and DF was statistically significant during the toxic stage, 30.21 (inter-quartile range 27.99–34.79) *vs* 40.61 (inter-quartile range 32.17–51.87) pg/ml, respectively ($p=0.019$). There was no significant difference in plasma VEGF levels between the DHF and DF patients at the febrile stage or during follow-up.

Changes in serum and plasma VEGF levels during the course of dengue virus infection

Fig. 3 demonstrates serial changes in serum and plasma VEGF during each stage of dengue virus infection. There were significant changes in serum VEGF ($p<0.001$) and plasma VEGF ($p=0.045$) during different stages of the disease. *Post-hoc* analysis showed that there was no difference in serum or plasma VEGF level between the

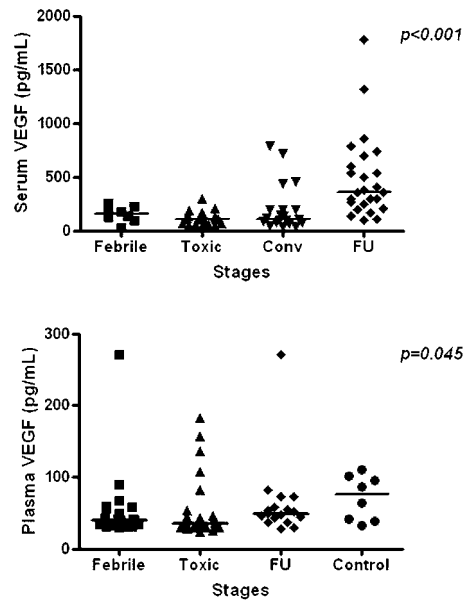

FIG. 3. Serum VEGF (top) and plasma VEGF (bottom) levels during the course of dengue virus infection. The horizontal lines indicate the median value for each group. Conv, convalescence; FU, follow-up.

febrile and toxic stages, but both serum and plasma VEGF were higher during recovery (at follow-up) than in the toxic stage ($p<0.05$).

Discussion

In DHF, vascular leakage and endothelial dysfunction are widely believed to be the main pathophysiological processes.[1,11] There are few data about the role of VEGF in the pathogenesis of vascular leakage in DHF. Initially, we hypothesised that VEGF might play a role in vascular leakage in DHF/DSS as one of its properties was to enhance vascular permeability.

The original concept was that serum VEGF levels at follow-up could be used as normal controls but the value (mean 486 pg/mL, median 352 pg/mL) appeared to be higher than normal values reported in other studies. Several studies have reported serum VEGF values in normal children.[12,13] Te Loo *et al.* reported a mean (SD) serum VEGF of 290 (130) pg/ml in 39 normal control children [age 2.9 (1.7) years],[12] and Ootaki *et al.* reported a mean value of 203 (221.6) pg/ml in 61 normal control children [aged 6.0 (3.4) years] compared with those with congenital cyanotic heart disease.[13] Because of a lack of normal control values and the possibility of a lower platelet count causing a spuriously low serum VEGF level in patients with DHF and DSS,[14,15] we additionally collected plasma samples to determine VEGF in patients with dengue virus infection and normal controls.

Serial determination of serum and plasma VEGF levels demonstrated no elevation of VEGF during the febrile or toxic stages in patients with dengue virus infection, and the levels in DHF patients were not higher than in DF patients. Contrary to our original hypothesis, both plasma and serum VEGF appeared to be lower in patients with more severe dengue virus infection. Our results differ from those of Tseng *et al.* who reported higher plasma VEGF levels in adults with DHF compared with DF and controls.[9] Srikiatkhachorn *et al.* observed a rise in the free but not total VEGF in the plasma of DHF patients along with a decline in the soluble form of VEGF receptor 2 (sVEGFR2) at the time of plasma leakage, followed by a decline in VEGF at 4–6-day follow-up with a rise of sVEGFR2. They concluded that VEGF regulated vascular permeability and its activity was controlled by binding to sVEGFR2.[10] If VEGF is indeed the cause of increased vascular permeability in dengue haemorrhagic fever, there are two possible reasons for the low plasma VEGF in this study. First, because of its small size (molecular weight 34–42 kDa, much less than the 66 kDa of albumin), plasma VEGF may be spuriously low because of its leakage from the intravascular compartment. Second, because the bound form of VEGF could not be detected by ELISA, incremental binding of VEGF to its receptor during the toxic stage might result in low plasma levels.

During the follow-up period, serum VEGF levels appeared to be increased. The reason for this is not clear, although it is possible that VEGF plays a role in the recovery phase of dengue infection. Further study is needed to confirm this finding.

Acknowledgments

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Increased level of hepatocyte growth factor in children with dengue virus infection

N. VORAPHANI*, A. THEAMBOONLERS*, A. KHONGPHATTHANAYOTHIN*, C. SRISAI*† & Y. POOVORAWAN*

**Department of Paediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand and*
†Hadyai Hospital, Songkhla, Thailand

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Abstract

Background: Evidence of hepatocellular damage is common in dengue-infected individuals. Hepatocyte growth factor (HGF), a key cytokine responsible for liver regeneration, may play a prognostic role in dengue virus infection.

Aim: To determine the relationship between serum HGF level and disease severity in patients with dengue virus infection.

Methods: Serum samples from 27 children [17 dengue fever (DF), ten dengue haemorrhagic fever (DHF)] with serologically confirmed dengue virus infection during the febrile, toxic stages and at follow-up were analysed for HGF. Serum samples obtained from nine healthy children served as the control group.

Results: In dengue-infected patients, serum HGF was significantly higher at the febrile and toxic stages than at follow-up ($p<0.05$). In comparison with DF, patients with DHF had a greater level of HGF at the febrile stage ($p<0.05$). A cut-off HGF level of 1220 pg/mL obtained during the febrile stage showed a sensitivity of 90% and a specificity of 53% for predicting clinical progression to DHF (area under the ROC curve 0.75).

Conclusion: Serum HGF level at the early stage of dengue virus infection is elevated and may be a useful predictor for clinical progression to DHF.

Introduction

Dengue virus infection is expanding globally and emerging as a major public health problem, principally in tropical countries, with an estimated annual incidence of 50–100 million cases. The clinical spectrum of dengue infection ranges from asymptomatic to dengue fever (DF), dengue haemorrhagic fever (DHF) and a severe, potentially fatal form, dengue shock syndrome (DSS).[1] The liver is one of the major target organs of dengue virus and hepatocellular damage can occur in varying degrees during the course of the disease.[2,3]

Hepatic dysfunction is common in dengue-infected individuals, including painful hepatomegaly and increased levels of the hepatic enzymes, aspartate transaminase (AST) and alanine transaminase (ALT). The elevation of liver enzymes, predominantly AST, is significantly greater in patients with DHF and DSS than DF, suggesting that the magnitude of liver injury is associated with disease severity.[2] Generally, the increase in transaminase levels is mild to moderate, reaching maximum values around the 9th day after onset of fever and gradually normalising thereafter within a few weeks.[3] Although the extent of liver involvement in patients with dengue virus infection is usually mild, fulminant

Corresponding author: Professor Yong Poovorawan, Center of Excellence in Clinical Virology, Department of Paediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand. Email: Yong.P@chula.ac.th

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hepatitis and liver failure are further complications of severe DHF/DSS, seen mainly in children and young adults, and are associated with a poor prognosis.[4,5]

The exact pathogenic mechanism of hepatocellular injury during dengue virus infection is not fully understood. It could be the direct effect of the virus on liver cells or the result of a dysregulated immune response to the virus.[6] Histopathological findings of fatal cases revealed centrilobular necrosis, microvesicular steatosis, Kupffer cell hyperplasia, mononuclear cell infiltration in the portal tract, and Councilman bodies, indicating an apoptotic process.[7,8] A range of pro-inflammatory and immunoregulatory cytokines as well as various chemokines have been studied in the pathogenesis of dengue virus infection.

Hepatocyte growth factor (HGF), a pleiotropic cytokine, has been recognised as the most potent mitogen for mature hepatocytes. This cytokine plays an essential role in liver regeneration after injury.[9] Increase in serum HGF has been observed in various liver diseases such as alcoholic hepatitis, fulminant hepatic failure and hepatocellular carcinoma.[10–13] In addition, it may serve as a predictor for disease severity and outcome. Although hepatic damage in dengue infection has been described frequently, the role of HGF in this illness has never been studied. This study was designed to determine the relationship between serum HGF levels and disease severity in children hospitalised with dengue infection.

Subjects and Methods

Study patients

From January 2005 to December 2006, children admitted to Had Yai Hospital, Songkhla with the initial diagnosis of dengue virus infection were enrolled. Patients aged 5–15 years and with serological confirmation of dengue virus infection were included. Those in whom any other acute/chronic diseases had previously been diagnosed were excluded, as were those who refused consent to the study and/or whose parents did so. The research protocol was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University. Written informed consent was obtained from an appropriate guardian and/or the patient prior to enrolment.

Blood sample collection

Upon receiving informed consent, blood samples were collected at three stages: febrile stage (1st day of enrolment), toxic stage (the day of defervescence) and recovery stage (at follow-up) for determination of serum HGF levels. Three ml of blood were collected and centrifuged at approximately 1000 G. The serum was then removed and stored at $\leqslant -70^{\circ}$C for subsequent analysis.

Determination of HGF level

HGF level was determined by a commercially available ELISA kit (Quantikine, R&D Systems, Minneapolis, MN, USA).

Determination of right pleural effusion

The amount of right pleural effusion was determined by ultrasonographic examination of the right pleural space. A transducer was placed at the right postero-lateral aspect of the chest wall, at the level of the xiphoid process. The maximum width of the pleural effusion was measured. Any pleural effusion would qualify the patient for diagnosis of DHF.

Clinical data

The progression of the patient's illness was classified into three clinical stages as febrile, toxic and convalescent according to World Health Organization (WHO) criteria.[1] The toxic stage was defined as the day of defervescence or the presence of haemoconcentration and/or shock. The convalescent stage was defined as 24–48 hours after the

toxic stage and once the patient was recovering. Follow-up was performed at least 1 week after the toxic stage. Acute and convalescent sera from all subjects were sent for serological confirmation of dengue virus. Dengue virus infection was diagnosed and classified as DF, DHF and DSS according to WHO case definitions.[1] Diagnosis of DHF required evidence of capillary leakage (rising of haematocrit >20% or presence of pleural effusion and/or ascites) on at least one occasion. In patients without a baseline haematocrit, the percentage of haemoconcentration was determined using the difference between maximum and minimum haematocrit during the same admission. DSS was diagnosed when a patient with DHF had clinical findings of shock such as: (i) hypotension in relation to age and cold, clammy skin or restlessness, or (ii) narrow pulse pressure (<20 mmHg) and rapid, weak pulse.[1] Classification of the patient's clinical severity was agreed by two physicians. All patients were treated by in-house staff and attending staff, according to WHO guidelines.[1]

Confirmation of dengue virus infection

Serological studies of dengue virus infection were performed at the Department of Medical Sciences, Ministry of Public Health (Nonthaburi, Thailand) using enzyme-linked immunosorbent assay (ELISA).

Data collection and analysis

Data were analysed using Prism for Windows, version 5 (GraphPad Software Inc., La Jolla, CA, USA). Continuous variables were expressed as mean (SD) and were compared using the *t*-test or one-way ANOVA. Categorical variables were compared by the χ^2 test or Fisher's exact test, as appropriate. A *p*-value of <0.05 was considered significant.

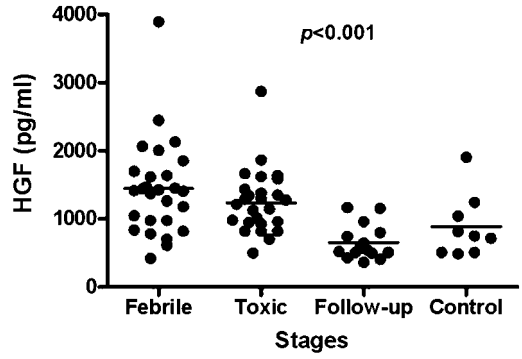

FIG. 1. HGF levels in children with dengue infection during different stages of the illness ($p<0.001$).

Results

Sixty-nine serum samples from 27 patients with serologically-confirmed dengue virus infection (17 with DF, ten with DHF) were analysed for serum HGF levels. Nine healthy children served as normal controls. Demographic and clinical data are summarised in Table 1.

HGF serum levels in patients with dengue virus infection (DF + DHF) during different stages of the disease and in controls are shown in Fig. 1. Serum HGF was significantly different between the three stages of the disease ($p<0.001$). *Post-hoc* analysis

TABLE 1. *Demographic and clinical data for 27 patients.*

	DF ($n=17$)	DHF ($n=10$)	*p*-value
Age (y)*	9.7 (2.4)	9.9 (2.8)	NS
M/F	9/8	9/1	NS
Body temperature max (C)*	39.6 (1.0)	39.7 (0.5)	NS
Maximum haematocrit (%)*	41.0 (3.9)	45.5 (4.0)	<0.01
Lowest platelet ($\times 10^9$/L)*	86 (40)	74 (70)	NS

* Mean (SD); NS, not significant.

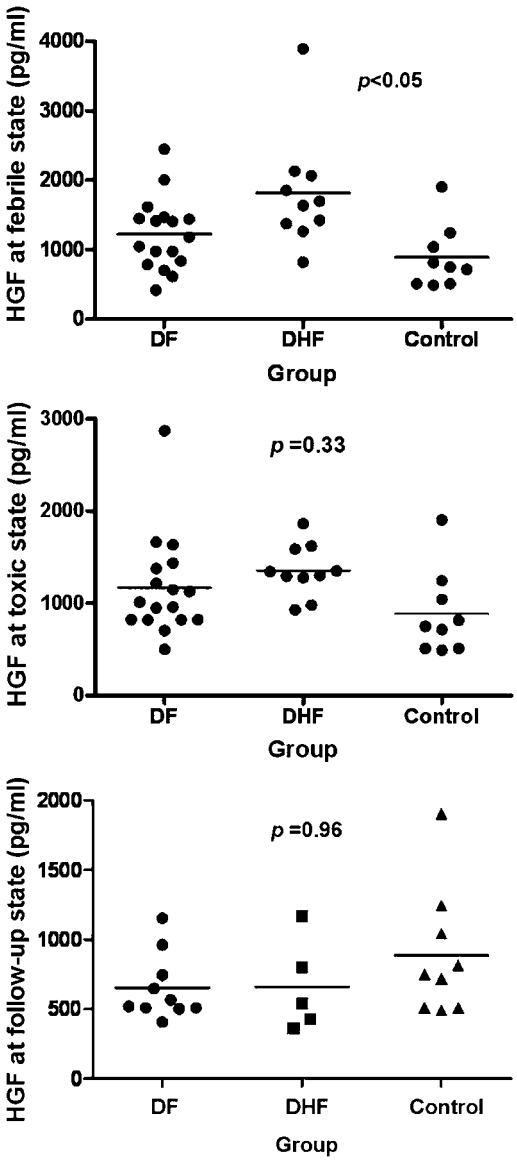

FIG. 2. HGF levels in patients with DF *vs* DHF at different stages of the illness.

showed that HGF levels in dengue-infected patients were significantly higher at the febrile and toxic stages than at follow-up ($p<0.05$).

Fig. 2 shows the difference between HGF levels in patients with DF and DHF at different stages of the disease. At the febrile stage, serum HGF was significantly different between DF and DHF patients (top figure, $p<0.05$). There was a tendency towards a higher level of serum HGF in patients with DHF than in those with DF at the toxic stage (centre figure, $p=0.33$), but not at follow-up (bottom figure, $p=0.96$). Receiver operating characteristics analysis showed that the area under the curve was 0.75 when using HGF levels at the febrile stage to predict clinical progression to DHF (Fig. 3). A cut-off serum HGF level of 1220 pg/mL obtained during the febrile stage showed a sensitivity of 90% and a specificity of 53% for predicting progression to DHF.

Ultrasonographic examination of the right pleural space was undertaken in 23 patients at the convalescent stage. Five children were found to have pleural effusion. There was no significant relationship by linear regression analysis between thickness of pleural effusion and serum HGF levels at the febrile and toxic stages ($r=0.26$, $p=0.22$ for febrile and $r=0.02$, $p=0.95$ for toxic stages).

Discussion

HGF is a heparin-binding polypeptide growth factor which is known to be the most potent trigger of hepatocyte regeneration following liver injury. This cytokine is synthesised by a range of cells including non-parenchymal liver cells such as sinusoidal wall endothelial cells, Kupffer cells and Ito cells.[15] Various studies conducted in patients with different types of infectious and non-infectious liver diseases, including hepatitis B, fulminant hepatic failure, and hepatocellular carcinoma, have demonstrated a variable degree of increased serum HGF levels.[12,13,16] Some research has also investigated its correlation with biochemical parameters of hepatic damage and supported its value as a prognostic marker.

Evidence of hepatocellular damage in dengue-infected patients, as indicated by raised transaminase levels (predominantly AST), has been described and was more pronounced in DHF than in DF.[2]

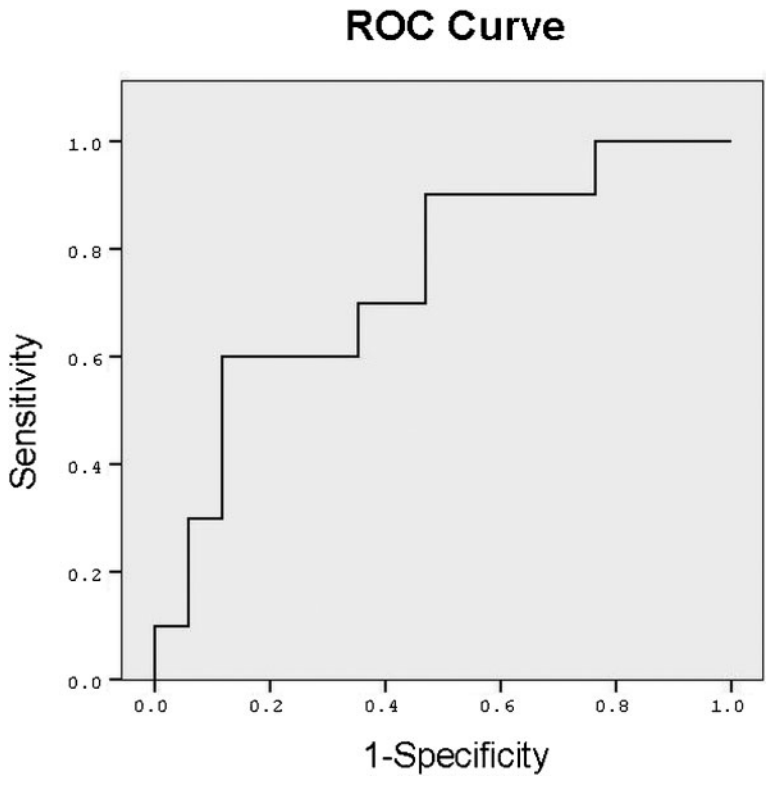

FIG. 3. Receiver operating characteristic (ROC) curve for using HGF during the febrile stage to predict clinical progression to DHF *vs* DF (area under the curve 0.75).

Nevertheless, the level of HGF during dengue infection has never been investigated. We initially hypothesised that elevated serum HGF levels might be associated with dengue virus infection and related to disease severity.

In accordance with the original hypothesis, our study demonstrated that serum HGF levels were significantly elevated during the febrile and toxic stages of dengue infection. Furthermore, DHF patients tended to have a higher HGF level than DF patients during the febrile stage (Fig. 2, top). Although HGF appeared to be higher in DHF than in DF at the toxic stage (Fig. 2, centre), the difference did not reach statistical significance, possibly owing to the relatively small sample size. The early increase in serum HGF levels observed in our study might have a prognostic value in a patient suspected of having dengue virus infection. A cut-off level of 1220 pg/mL obtained during the febrile stage was found to be a sensitive predictor of clinical progression to DHF (sensitivity 90%). Therefore, serum HGF obtained at the febrile stage might become a valuable tool for early identification of dengue-infected patients at risk of developing DHF and should be further studied. Owing to small sample sizes, the relationship between HGF and severity of pleural effusion was not statistically significant. Additional studies with more subjects are required to better elucidate this finding.

The mechanism underlying increased HGF levels in patients with dengue infection, particularly DHF, has remained unclear. We postulate that it might be owing to enhanced production of this hepatotropic factor in response to dengue-induced liver injury, which has previously been reported

to be more profound in DHF than in DF.[2] In addition, since the liver is responsible for clearance of HFG, decreased elimination as a result of liver cell damage might be another explanation. Additional investigations are required to elucidate the possible mechanisms and the significance of HGF elevation in dengue infection.

We provide preliminary data suggesting that serum HGF level at the early stage of dengue virus infection is elevated and may serve as a useful predictor of clinical progression to DHF. Further studies with larger patient groups are warranted to confirm these encouraging results.

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